THE IMPACT OF PATHOGEN-PATHOGEN AND HOST-PATHOGEN SIGNALING IN LARVICULTURE OF THE GIANT FRESHWATER PRAWN (*MACROBRACHIUM ROSENBERGII*)

PANDE GDE SASMITA JULYANTORO

Thesis submitted in fulfillment of the requirements for the degree of
Doctor (PhD) in Applied Biological Sciences
2015

Dutch translation of the title:
De impact van pathogeen-pathogeen en gastheer-pathogeen communicatie op de larvicultuur van de reuzen zoetwatergarnaal (*Macrobrachium rosenbergii*)

Cover page: photograph of the giant freshwater prawn (*Macrobrachium rosenbergii*) was taken by Okky Amalia.

To cite this work:

G. S. J. Pande (2015) The impact of pathogen-pathogen and host-pathogen signaling in larviculture of the giant freshwater prawn (*Macrobrachium rosenbergii*). PhD thesis, Ghent University, Belgium.

ISBN:

This work was funded by the Directorate General of Higher Education of Indonesia/DIKTI through three years doctoral grant to Pande GSJ and Bijzonder Onderzoeksfonds (BOF)-special research fund for finalizing PhD (Code : 01DI0114) from Ghent University.

Promotoren:
Prof. dr. ir. Peter Bossier
Laboratory of Aquaculture and *Artemia* Reference Center
Department of Animal Production
Faculty of Bioscience Engineering
Ghent University

Dr. ir. Tom Defoirdt
Laboratory of Aquaculture and *Artemia* Reference Center
Department of Animal Production
Faculty of Bioscience Engineering
Ghent University

Prof. dr. ir. Marc Van Meirvenne (Acting Dean)

Rector:
Prof. dr. Anne De Paepe

Members of the examination and reading committee (*):

Prof. dr. ir. Peter Bossier
Department of Animal Production
Faculty of Bioscience Engineering, Ghent University, Belgium

Dr. ir. Tom Defoirdt
Department of Animal Production
Faculty of Bioscience Engineering, Ghent University, Belgium

Em. Prof. dr. Patrick Sorgeloos
Department of Animal Production
Faculty of Bioscience Engineering, Ghent University, Belgium

Dr. Massimo Marzorati*
Department of Biochemical and Microbial Technology
Faculty of Bioscience Engineering, Ghent University, Belgium

Prof. dr. Annemie Decostere*
Department of Morphology
Faculty of Veterinary Medicine, Ghent University, Belgium

Dr. Øivind Bergh*
Institute of Marine Research
Bergen, Norway

Prof. dr. ir. Sven Mangelinckx
Department of Sustainable Organic Chemistry and Technology
Faculty of Bioscience Engineering, Ghent University

Prof. dr. ir. Mia Eeckhout (Chairman)
Department of Applied Biosciences
Faculty of Bioscience Engineering, Ghent University, Belgium

Whatever has happened, has happened for good
Whatever is happening is also for good
Whatever will happen, shall also be good

What have you lost that you cry for?
What did you bring, that you have lost?
What did you create that was destroyed?
What you have taken, has been from here
What you gave has been given here

What belongs to you today?
Belonged to someone yesterday
And will be someone else's tomorrow

Change is the law of the Universe

-The Bhagavadgita-

This work is dedicated to my family, my parents and all people who initiated and encouraged me to learn and explore new things that would help me grow as a human being

NOTATION INDEX

AHL	*N*-acylhomoserine lactone
AHPND	Acute hepatopancreatic necrosis disease
AI-2	Autoinducer-2
ANOVA	Analysis of variance
BHL	*N*-butanoyl homoserine lactone
CAI-1	Cholerae Autoinducer-1
CFU	Colony forming unit
DAHL	*N*-(α,α-dibromoacetyl) homoserine lactone
DBHL	Dibrominated *N*-3-oxohexanoyl homoserine lactone
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
DO	Dissolved oxygen
DOPA	Dopamine
EMS	Early mortality syndrome
FAO	Food and Agriculture Organization
HAI-1	Harveyi Autoindicer-1
HHL	*N*-hexanoyl-L-homoserine lactone
MBHL	Monobrominated *N*-3-oxohexanoyl homoserine lactone
NE	Norepinephrine
ND	Not Detected
OD	Optical density
OHHL	*N*-3-oxohexanoyl homoserine lactone
PCR	Polymerase chain reaction
PL	Post Larvae
QS	Quorum sensing
QSI	Quorum sensing inhibitor(y)
RifR	Rifampicin resistant
RNA	Ribonucleic acid
USD	US dollar

CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

INTRODUCTION

INTRODUCTION

Aquaculture comprises the farming of aquatic organisms, including fish, mollusks, crustaceans and aquatic plants (FAO, 1988). This activity serves a variety of purposes, not only growing aquatic species used for human consumption, but also for cultivating ornamental species for aquarium trade as well as producing some aquatic species for pharmaceutical or nutraceutical products. As the fastest growing food producing sector (Subasinghe, et al., 2009; Bostock et al., 2010), aquaculture plays an important role in the economic development worldwide, especially in many developing countries.

World aquaculture production increased from 32.4 million tons in 2000 to 66.6 million tons in 2012, and covers almost half of the world fish and shellfish supply (FAO, 2014). Increasing aquaculture production is required to fill the gap between demand and supply of (aquatic) protein source for human consumption, given the steady state of capture fisheries production in the last two decades (**Figure 1**). Aquaculture production is expected to grow by 33 percent, to about 79 million tons, by 2021. In 2012, the production was still dominated by freshwater species which shared about 41.9 million tons, whereas 24.7 million tons (excluding aquatic plants) were coming from marine aquaculture (FAO, 2014).

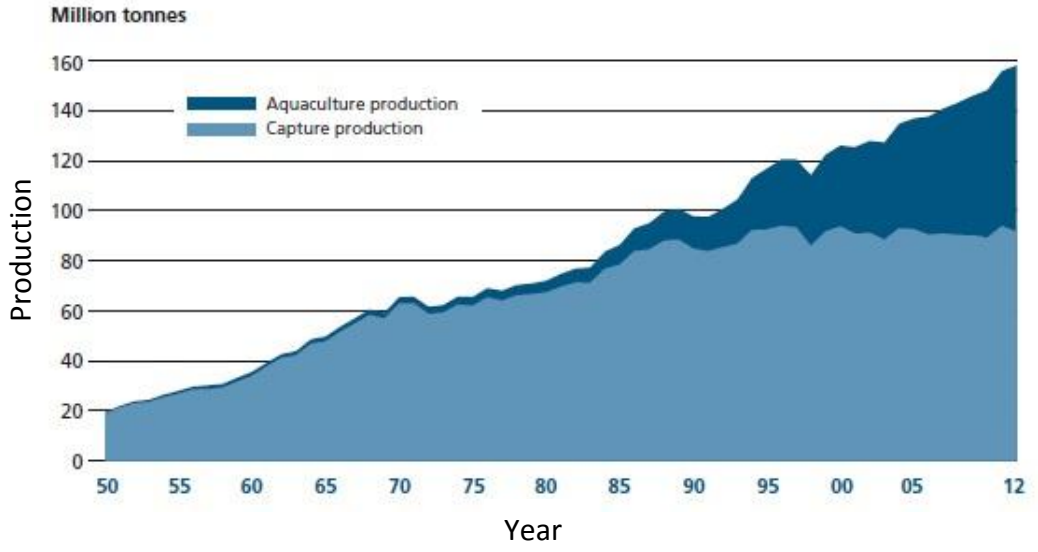


Figure 1. World capture fisheries and aquaculture production (FAO, 2014)

Nominally, world trade of fish and fish products increased from $8 billion in 1976 to $128 billion in 2012, which in real terms translates into an average annual growth rate of 4.0 percent (FAO, 2012). Developing countries are well integrated in the global seafood trade, with more than 54 percent of all fishery exports by value and more than 60 percent by quantity (in live weight equivalent) coming from developing countries (FAO, 2012). According to FAO, since the mid-1990s, production from global capture fisheries has stabilized around 90 million tons (**Figure 1**). With this stagnating capture fishery production and an increasing human population, aquaculture is perceived as having the greatest potential to assure food security in the future.

The World Bank reported that during the past three decades, global aquaculture production expanded at an average annual rate of more than 8 percent, from 5.2 million tons in 1981 to 62.7 million tons in 2011 (World Bank report, 2013). Aquaculture's contribution to total food fish supply grew from 9 percent in 1980 to 48 percent in 2011 (FAO, 2013). The estimated number of fish farmers also grew from 3.9 million in 1990 to 16.6 million in 2010. The rapid and massive growth of aquaculture production has contributed significantly to increased production of species whose supply would be otherwise constrained given the lack of growth in capture fisheries production. As a result, the prices of these species (for example, salmon and shrimp) declined, especially during the 1990s and in the early 2000s (FAO, 2012). The global aquaculture production during 1950-2010 is depicted in **Figure 2**.

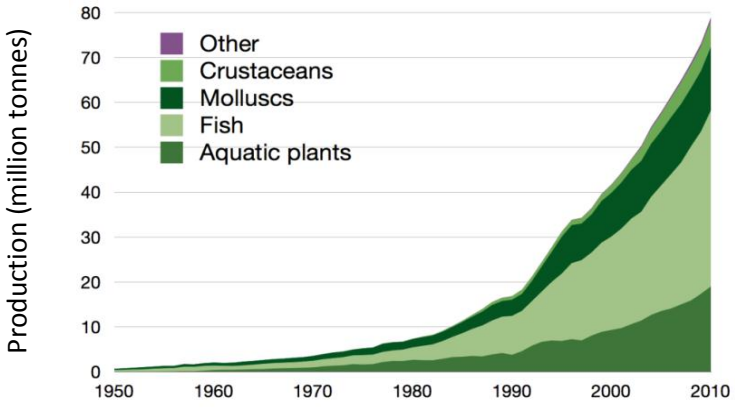


Figure 2. Global aquaculture production (FAO, 2012)

INFECTIOUS DISEASES AND ANTIBIOTIC USE IN AQUACULTURE

Despite the massive development of intensive aquaculture worldwide, this sector still faces important challenges with respect to infectious diseases. The aquatic environment is more supportive to pathogenic bacteria independently of their host than the terrestrial environment and consequently, pathogens can reach high densities around the animals, which then ingest them either with the feed or when they are drinking. This results in highly unpredictable survival rates, especially in the larval stages of several aquaculture animals (Verschuere et al., 2000). Estimates by the FAO of global disease losses in aquaculture are in the range of several billion USD per year, which is approximately 15% of the value of world farmed fish and shellfish production (Subasinghe et al., 2001). For example, losses up to USD 100 million caused by luminescent vibriosis outbreak were reported in shrimp hatcheries in Indonesia in 1991 (Defoirdt, 2007) and combined estimated losses from 11 countries for the period 1987-1994 were reported to be as high as USD 3 billion (Israngkura & Sae-Hae, 2002). A recent disease outbreak in Penaeid shrimp culture, the early mortality syndrome (EMS) or acute hepatopancreatic necrosis disease (AHPND) also resulted in huge loses in the Asian shrimp aquaculture industry. The Global Aquaculture Alliance has estimated that losses to the Asian shrimp culture sector amount to USD 1 billion per year (De Schryver et al., 2014).

In attempts to cure and prevent infectious diseases, some chemical compounds were used in aquaculture practices, including disinfectants (e.g. hydrogen peroxide and malachite green), anthelmintic agents (e.g. pyrethroid insecticides and avermectins) and most importantly antibiotics (e.g. sulfonamids and tetracyclines) (Rawn et al., 2009). Antibiotics are still critically important as a first line therapy for the treatment of various bacterial infections in aquaculture (Defoirdt, 2013b). There are three methods by which antimicrobial agents can be administered to aquatic animals: medicated feed, immersion and injection (Smith, 2008). However, there are little hard data that allow an authoritative statement to be made about the relative frequencies with which each of the three methods is used in global aquaculture (Smith, 2008).

The most common route for delivery is through mixing in formulated feed. This method still is less effective than injection because aquaculture animals, especially fish, do not effectively metabolize antibiotics and will pass them largely unused back into the environment. It has been estimated that 75 percent of the antibiotics fed to aquaculture animals are excreted into the water (Burridge et al., 2010). These residuals will remain in the sediment, exerting selective pressure, thereby altering the composition of the microflora of the sediment and selecting for antibiotic-resistant bacteria (Cabello, 2006). A recent investigation regarding to antibiotic use in aquaculture was conducted in an EMS/AHPNS affected area in Soc Trang province of Vietnam in July of 2013 (Tran et al., 2015). Further, Tran et al. (2015) reported that in this area antibiotics were used intended to reduce losses due to EMS/AHPNS in shrimp farms since 2012. Among several antibiotics that were being used in shrimp farms, oxytetracycline is the most common due to its affordable price and market availability. Farmers' observation has shown that routine use of antibiotics has quickly lost its effectiveness since antibiotics were first launched for use in shrimp pond to cure EMS/AHPNS in 2012. The laboratory analyses could purify several isolates of both bacteria causing EMS/AHPNS (pathogenic) and non-pathogenic *Vibrio parahaemolyticus*. Subsequent antiobiotic sensitivity tests have shown that 100% of non-pathogenic *V. parahaemolyticus* isolates and 85.7% of pathogenic bacterial isolates of 2013 was resistant to oxytetracycline. Meanwhile, 100% isolates of of both pathogenic and non-pathogenic *V. parahaemolyticus* isolated in 2011 and 2012 was sensitive to oxytetracycline. This is a strong indication that *Vibrio parahaemolyticus* causing EMS/AHPNS can develop resistance to oxytetracycline in such a short period of time.

As antibiotic resistance genes are often located on mobile genetic elements, such as on transferable plasmids and integrons in pathogenic *Aeromonas* spp., *Citrobacter* spp., *Edwardsiella* spp., *Photobacterium* spp. and *Vibrio* spp. (Sorum, 2006; Ishida et al., 2010). Tran et al. (2015) also revealed that avirulent *Vibrio parahaemolyticus* isolated from infected shrimp were also resistant to oxytetracycline, posing a concern of transfering of antibiotic resistance via mobile genetic elements among bacterial strains. The resistance

gene determinants of these aquatic antibiotic-resistant bacteria have the potential of being transmitted by horizontal gene transfer, not only to other aquatic bacteria, but also to terrestrial bacteria including human and animal pathogens, as has been reported for *Salmonella enterica* serotype Typhimurium and *Vibrio cholerae* (Cabello, 2006; Sørum, 2006; Defoirdt et al., 2011a).

Instead of some countries generally those of Northern Europe, North America and Japan which have a stict regulation on the use of antimicrobial agents in aquaculture, it is almost impossible to obtain a complete overview of the dosage and type of compounds used due to a lack of data (Heuer et al., 2009; Smith, 2008). For instance, one study in Thailand, in the year 2000 reported that from seventy-six farmers interviewed, 74% used antibiotics where most used by prophylactically, some on a daily basis, and at least thirteen different antibiotics were used including chloramphenicol, gentamycin, trimethoprim, tiamulin, tetracyclines, quinolones and sulfonamids (Holmström et al., 2003). It is also difficult to determine the amounts of antibiotics used because different countries have different regulations for using antibiotics (Burridge et al., 2010). The rare available estimates indicate that there is a large variation in the amount of antibiotics used in different countries, ranging from 1 gram per metric ton of production in Norway to 700 gram per metric ton in Vietnam (Smith, 2008).

In addition to selection for resistance, another problem is the (potential) presence of residual antibiotics in commercialized aquaculture products. The use of antibiotics has led to undetected consumption of those antibiotics by humans who regularly consume aquaculture products (Cabello, 2006). This can generate problems of allergy and toxicity, which are difficult to diagnose because lack of information on antibiotic content of the aquaculture product (Alderman & Hastings, 1998). The problems related to antibiotic use in aquaculture resulted in a drastic restriction of the use of antibiotics in aquaculture in many countries (Cabello, 2006, Defoirdt et al., 2011a). Some examples of antibiotics used in aquaculture and examples of (multi)resistant aquaculture pathogenic bacteria are listed in **Table 1**.

Table 1. The different classes of antibiotics used in aquaculture and examples of (multiple) resistant aquaculture pathogenic bacteria (Adapted from Defoirdt et al., 2011a).

Class	Example	(Multiple) resistant bacteria	Source	Country
Aminoglycosides	Streptomycin	*Edwardsiella ictaluri*	Diseased striped catfish (*Pangasianodon hypophthalmus*)	Vietnam
Amphenicols	Florfenicol	*Enterobacter* spp. and *Pseudomonas* spp.	Freshwater Salmon farms	Chile
Beta-lactams	Amoxicillin	*Vibrio* spp. *Aeromonas* spp. and *Edwardsiella tarda*	Different aquaculture facility	Australia
Beta-lactams	Ampicillin	*Vibrio harveyi*	Shrimp farm and coastal waters	Indonesia
Fluoroquinolones	Enrofloxacin	*Tenacibaculum maritimum*	Diseased turbot (*Scopthalmus maximus*) and sole (*Solea senegalensis*)	Spain and Portugal
Macrolides	Erythromycin	*Salmonella* spp.	Marketed fish	China
Nitrofurans	Furazolidones	*Vibrio anguillarum*	Diseased sea bass and sea bream	Greece
Nitrofurans	Nitrofurantoin	*Vibrio harveyi*	Diseased penaeid shrimp	Taiwan
Quinolones	Oxolinic acid	*Aeromonas* spp. *Pseudomonas* spp. and *Vibrio* spp.	Pond water, pond sediment and tiger shrimp (*Penaeus monodon*)	Philippines
Sulphonamides	Sulphadiazine	*Aeromonas* spp.	Diseased katla (*Catla catla*), mrigel (*Cirrhinus mrigala*) and punti (*Puntius* spp.), India	India
Tetracyclines	Tetracycline	*Aeromonas hydrophila*	Water from mullet and tilapia farms	
Tetracyclines	Oxytetracycline	*Aeromonas salmonicida*	Atlantic salmon (*Salmo salar*) culture facilities	Canada

THE GIANT FRESHWATER PRAWN *MACROBRACHIUM ROSENBERGII*

One of the commercially important freshwater species is the giant freshwater prawn *Macrobrachium rosenbergii* (de Man, 1879). This species is the largest species in the genus *Macrobrachium,* and is native to Southeast Asia, Southern Pacific countries, northern Oceania, and western Pacific islands (New & Singholka, 1982). The external features of this species are shown in **Figure 3**. Anatomically, the body of *M. rosenbergii* is divided into two parts, cephalothorax and abdomen. The cephalothorax ends in a long and pointed rostrum with two pairs of antennae on both sides that are used for sensory reception. It has ten pairs of appendages or legs, the first five pairs are called pereiopods (walking legs) and the last five (at the abdomen) are swimming legs (pleopods). The abdomen is divided into six segments, the first five of which contain the pleopods, and the last segment (telson) is flanked by a pair of uropods.

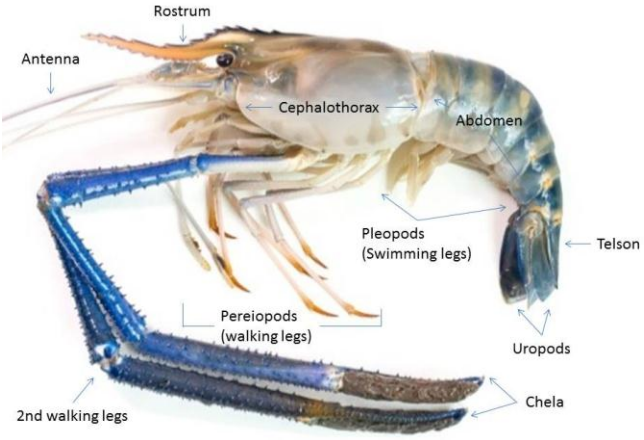


Figure 3. Morphology of *Macrobrachium rosenbergii*

There are four distinct phases in the life cycle of *M. rosenbergii*: egg, larva (zoea), postlarvae (PL) and adult. The larval phase is divided into 11 distinct stages and it takes 22-35 days to complete all larval stages to become post-larvae (PL). Similar to other crustaceans, freshwater prawn also undergo molting throughout their life cycle for growth. These animals require brackish water at the initial stages of their life and then the post-larvae move to freshwater, where they grow to adult.

The modern culture of the giant freshwater prawn *M. rosenbergii* has started in the early 1960s after the FAO expert Shao-Wen Ling from Malaysia and his colleagues documented the fundamental aspects of all developmental stages and the success story of commercial-scale hatchery production by Takuji Fujimura in Hawaii (New & Valenti, 2000). The culture of *M. rosenbergii* has expanded enormously since 1980, and in 2009, the global production reached a level of up to 80 times the production reported in 1980; i.e. about 200 thousand tons per year (New & Nair, 2012; FAO, 2014). However, *M. rosenbergii* production still faces some constraints, reflected by the stagnant and even slightly declining yields in some recent years (**Figure 4**).

Figure 4. Global aquaculture production of *Macrobrachium rosenbergii* (Source: FAO FishStat http://www.fao.org/fishery/species/2608/en)

One of those constraints is the low availability of high quality and healthy larvae of *M. rosenbergii*. This problem is often linked with bacterial disease outbreaks (Nhan, 2009). Given the opportunistic nature of many pathogens, there are some trigger factors such as high stocking density, poor water quality, temperature and pH changes, turbidity, ammonium and nitrite levels, inadequate nutrition and poor sanitation that cause physiological stress and/or physical injury to the animals, making them more susceptible to diseases. The most common bacterial diseases in *M. rosenbergii* are listed in **Table 2**.

Tabel 2. Bacterial diseases in *Macrobrachium rosenbergii* culture.

Disease	Causative agent(s)	References
Tail rot disease	*V. parahaemolyticus, V. anglinotenus, P. aeruginosa, Aeromonas* spp*., S. aureus*	Dinakaran et al., 2013
Muscle necrosis	*Enterococcus*-like bacterium closely related to *Enterococcus seriolicida*	Cheng & Chen, 2002
Shell disease, brown/black spot, black rot/erosion, blisters, necrosis of appendages	*Vibrio* spp., *Pseudomonas* spp*., Aeromonas* spp., chitinoclastic bacteria	Dinakaran et al., 2013; Pillai & Bonami, 2012; El-Gamal et al., 1986; Dugan & Frakes, 1973
Bacterial necrosis	Mixed bacterial infection: *Leucothrix* spp., and non-filamentous bacilli and cocci (*Pseudomonas* spp.); probably a secondary infection;	Pillai & Bonami, 2012; New et al., 2010; Tonguthai, 1997; FAO, 2014
White post larval disease	*Rickettsia* spp.	FAO, 2014, De Silva et al., 1989; Lacroix et al., 1994
Vibriosis	Non-luminescent vibrios, *V. cholerae*, *V. alginolyticus*,*V. harveyi* and *V. mimicus*	New et al., 2010; Oanh et al., 2001;
Enterococcosis	*Enterococcus faecium* associated with the yeast *Metschnikowia bicuspidate*	Chen et al., 2003; Pillai & Bonami, 2012
White muscle disease	*Lactococcus lactislactis*	Wang et al., 2008
Filamentous bacterial disease	*Leucothrix* spp.	Shailender et al*.,* 2012; Sandifer et al., 1975
Luminous bacterial diseases or luminescent larval syndrome	*V. harveyi* and closely related bacteria such as *V. campbellii* and *V. parahaemolyticus*	New et al., 2010; Tonguthai, 1997

Vibriosis causes significant losses in the aquaculture industry worldwide and is also one of the most important bacterial diseases in *M. rosenbergii* culture (Austin & Zhang, 2006; Ruwandeepika et al., 2012). Several studies documented that *Vibrio* spp. are a major cause of diseases (Tonguthai, 1997; Kennedy et al., 2006; New et al., 2010). In general, vibriosis is prevalent in the early life stages (eggs, larvae, and postlarvae) of giant freshwater prawn (Bhat & Singh, 1999), all of which need brackish water (New et al., 2010). One of the species that has been isolated from affected giant freshwater prawn larvae are luminous bacteria *Vibrio campbellii* (Tonguthai, 1997). The *Vibrio campbellii* BB120 (= ATCC BAA-1116) previously was designated as *Vibrio harveyi* (Lin et al., 2010). *Vibrio campbellii* and closely related bacteria are belonging to the *Harveyi* clade or *Vibrio* core group (Sawabe et al., 2007). It is very difficult to differentiate between species in this clade since they share high phenotypic and genotypic homology (Cano-Gomez et al., 2009). Sequence similarities for the 16S rRNA gene are high (>97.6%; Sawabe et al., 2007) and DNA–DNA re-association values are very close to 70% for most species pairs (Pascual et al. 2010). Some of the members of this clade have been documented as important pathogen of aquatic organisms which cause multibillion-dollar losses to the aquaculture industry (Thompson et al., 2004; Austin & Zhang 2006; Ruwandeepika et al., 2012)

ALTERNATIVE STRATEGIES TO CONTROL DISEASE IN AQUACULTURE

Because of the negative consequences related to antibiotic use in aquaculture, there are some intensive studies on the development of alternative methods to protect aquaculture animals from pathogenic bacteria. A holistic approach, which includes pathogen, host and environment (**Figure 5**), will probably be most effective in the long term (Defoirdt et al., 2011a). Since it would be unrealistic to expect that infection will be prevented in all case, new biocontrol strategies to control the pathogenic bacteria (together with the rational use of antibiotics) are needed to support the sustainability of aquaculture (Defoirdt et al., 2011a).

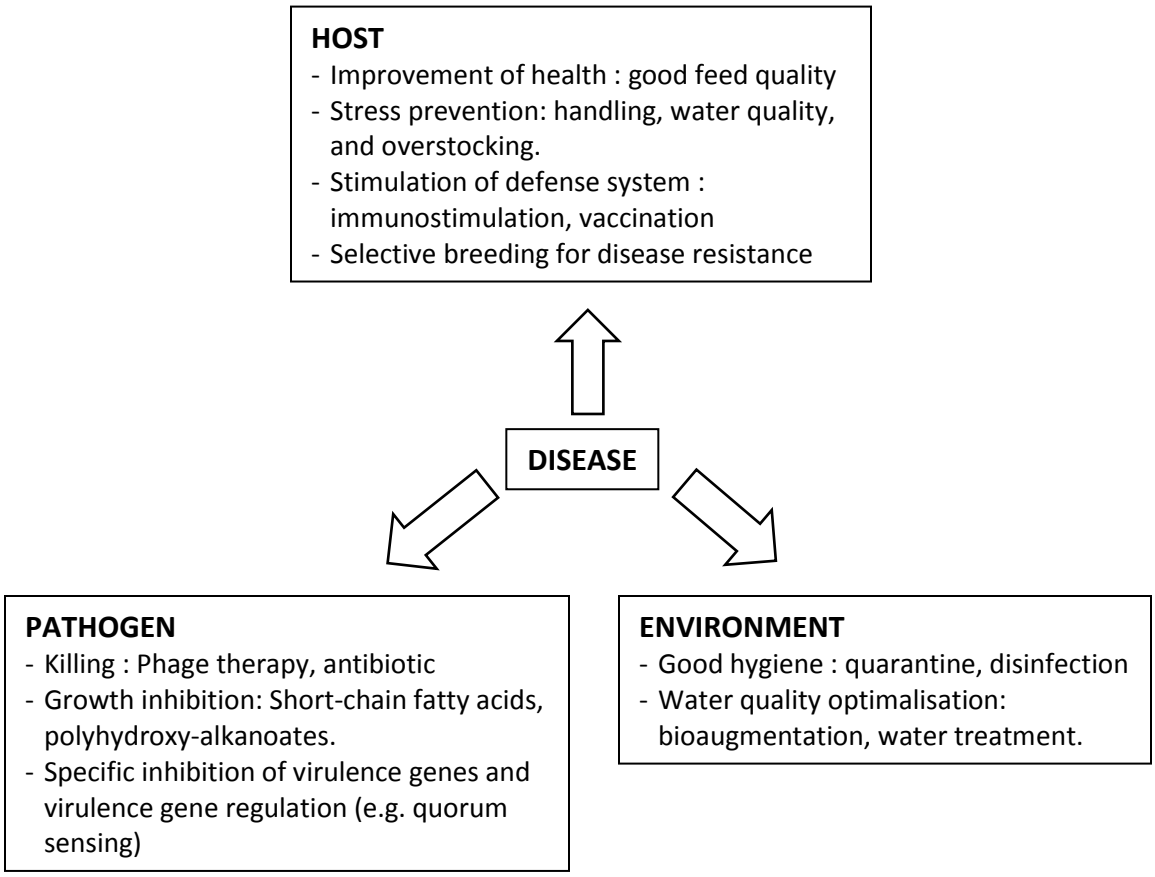


Figure 5. Strategies to prevent and control bacterial disease in aquaculture (Redrawn after Defoirdt et al., 2011a).

One alternative strategy to antibiotic in order to control bacterial diseases in aquaculture is by preventing the pathogenic bacteria from attacking the host without the need to kill them, called antivirulence therapy (Clatworthy et al., 2007). This strategy includes interference of pathogen-pathogen or host-pathogen signaling, which will be discussed in detail in **Chapter 2**.

CHAPTER 2

PATHOGEN-PATHOGEN SIGNALING AND HOST-PATHOGEN SIGNALING: A LITERATURE REVIEW

ABSTRACT

The increasing occurrence of antibiotic-resistant bacteria has created an adverse situation in the aquaculture sector. Although still being the first line therapy in aquaculture, antibiotics are becoming less effective in treating bacterial diseases in some cases. Furthermore, the use of antibiotics is being reduced because of environmental and human health concerns, and consequently, alternative strategies to control bacterial diseases are urgently needed. Antivirulence therapy aims to prevent the pathogenic bacteria from attacking the host by controlling virulence (regulatory) mechanisms. This strategy requires a thorough understanding of bacterial pathogenicity mechanisms to find suitable targets for novel therapeutics. Some strategies have been developed such as interfering with host-pathogen signaling or inhibiting of quorum sensing (QS), bacterial cell-to-cell communication. The major advantage of antivirulence therapy is that in principle it only interferes with bacterial virulence without any strong pressure on bacterial growth and proliferation, resulting in a lower risk of resistance development. This review briefly summarizes the problems related to antibiotic usage in aquaculture, followed by giving an overview of the current knowledge on some major bacterial pathogenicity mechanisms in aquaculture pathogens, with a focus on host-pathogen signaling and quorum sensing.

Keywords: Antibiotic resistant, quorum quenching, quorum sensing, vibriosis, virulence factors

VIRULENCE MECHANISMS OF BACTERIAL AQUACULTURE PATHOGENS

Virulence factors

Generally, infection by pathogenic bacteria requires the production of different virulence factors, gene products that allow the pathogen to enter and damage their target host. Major virulence factors include gene products involved in bacterial motility, adhesion, host tissue degradation, iron acquisition, secretion of toxins, and protection of host defense (Defoirdt, 2013b). The role of different virulence factors are listed in **Table 3**.

Table 3. Examples of virulence factors in pathogenic bacteria.

Virulence factor	Role	References
Motility (swimming and swarming), adhesion and chemotaxis.	Colonization of host surfaces	Finlay & Falkow 1997; McCarter, 2001; McCarter, 2004; Yang & Defoirdt, 2014.
Production of extracellular polysaccharides (EPS) and biofilm formation.	Colonization of host surfaces and protection against the defense system of the host	Hsieh et al., 2003; Chen et al., 2010; Mah & O'Toole 2001; Donlan & Costerton, 2002
Production of lytic enzymes including haemolysins, proteases, lipases and chitinases.	Degradation of host tissues, thereby allowing the pathogen to obtain nutrients and to spread through tissues	Finlay & Falkow, 1997

Moreover, iron acquisition mechanisms are needed to thrive within the iron-depleted environment of a host, and many pathogenic bacteria can acquire iron by means of siderophores. Siderophores are low molecular weight iron-binding complexes secreted by bacteria and enabling bacteria to compete with iron sequestration by transferrin and lactoferrin (Bullen & Griffiths, 1999). Upon removing iron from host proteins, iron-loaded siderophores are bound by cognate receptors expressed at the bacterial surface and the siderophore–iron complex is then internalized into the bacterium and the iron is released for use as a nutrient source (Skaar, 2010). The importance of siderophores to bacterial

virulence is demonstrated by the decreased fitness of siderophore-defective strains in animal models of infection (Ratledge & Dover, 2000).

Furthermore, to establish a successful pathogen–host interaction, virulence proteins of the pathogen need to be transported to the bacterial surface, to the extracellular environment or directly into the host cell (De Buck et al., 2007). For extracellular secretion or direct secretion into host cells, various specialized secretion system (named type I–V) exist in Gram-negative bacteria, each responsible for the transport of a specific subset of proteins, however it has been noticed that not all Gram-negative bacteria possess all types of secretion systems (Gerlach & Hensel, 2007).

Regulatory mechanisms that control virulence factor production

Pathogenicity appears not to be species-dependent, but rather strain characteristic as some strains belonging to one specific species can be highly virulent, whereas other strains are avirulent (Ruwandeepika et al., 2012). The relationship between the presence of virulence genes and the pathogenicity of bacteria is not always evident (Cano-Gomez et al., 2009). The production of virulence factors is usually under strict regulatory control, including cell-to-cell communication (quorum sensing) and sensing of host factors such as catecholamine stress hormones (Natrah et al., 2011b; Ruwandeepika et al., 2012).

Pathogen-pathogen signaling (Quorum sensing)

Quorum sensing is a regulatory mechanism in which bacteria coordinate the expression of certain genes in response to the presence of small signal molecules called autoinducers. This mechanism was first discovered in the luminous marine bacterium *Vibrio fischeri* (Fuqua et al., 1996; Miller & Bassler, 2001) which lives in symbiotic association with the Hawaiian bobtail squid *Euprymna scolopes* (Nealson et al., 1970). It was thought that this kind of mechanism was restricted to a few species, until similar systems were found in many other bacteria (Bassler, 1999).

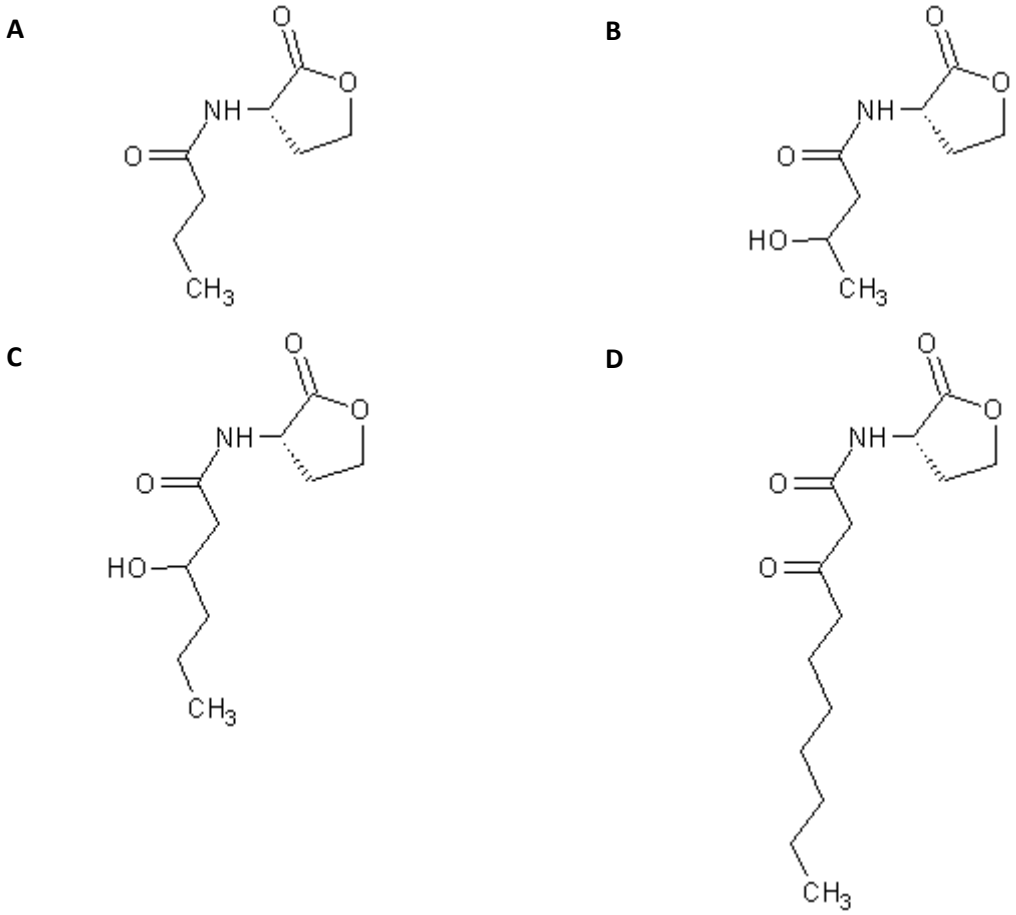
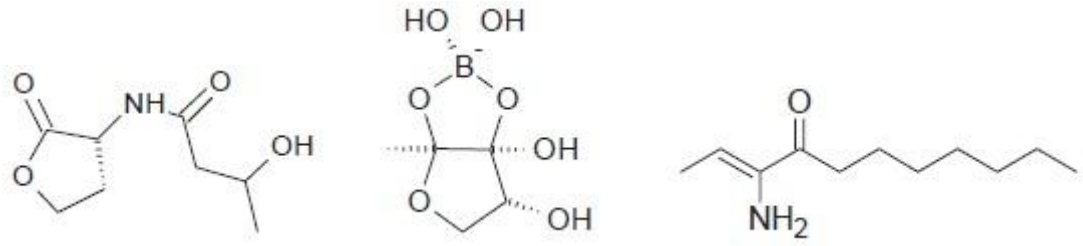


Figure 6. Structure of AHL molecules produced by different aquaculture pathogenic bacteria species: (**A**) *N*-butanoyl-$_L$-homoserine lactone produced by *Aeromonas hydrophila* and *Aeromonas salmonicida*, (**B**) *N*-(3-hydroxybutanoyl)-$_L$-homoserine lactone produced by *Vibrio campbellii*, (**C** and **D**) *N*-(3-hydroxyhexanoyl)-$_L$-homoserine lactone and *N*-(3-oxodecanoyl)-$_L$-homoserine lactone, both produced by *Vibrio anguillarum*.

There are two general types of quorum sensing systems in Gram-negative marine pathogens, i.e. systems that use N-acylhomoserine lactones (AHLs) as signal molecules and multi-channel signaling systems (Defoirdt et al., 2011a). AHLs of different species typically contain an invariable lactone ring connected to a variable acyl side chain with 4 and 18 carbons and this acyl chain can have an oxo or hydroxyl substitution at the third position (see **Figure 6**). Some aquaculture pathogens that use AHLs as signal molecule include *Aeromonas hydrophila*, *Aeromonas salmonicida*, *Edwardsiella tarda*, *Yersinia ruckeri* and *Vibrio anguillarum* (Natrah et al., 2011a).

Multi-channel quorum sensing systems are found in vibrios such as *Vibrio harveyi* and *Vibrio vulnificus* (Milton, 2006). The quorum sensing system in *Vibrio campbellii* strain BB120 (=ATCC BAA-1116) which was previously designated as *Vibrio harveyi* (Lin et al., 2010), is one of the most intensively studied systems. This bacterium uses a multi-channel quorum sensing system mediated by three different quorum sensing signal molecules that feed a shared phosphorylation/dephosphorylation signal transduction cascade that controls the production of master regulators, which in turn controls the expression of the genes responsible for bioluminescence and other traits (Henke & Bassler, 2004). The three channels are fed by the three small signal molecules including Harveyi autoinducer 1 (HAI-1), Autoinducer 2 (AI-2) and Cholerae autoinducer 1 (CAI-1) (**Figure 7**). *Vibrio campbellii* HAI-1 is an AHL, *N*-(3-hydroxybutanoyl)-$_L$-homoserine lactone) (Cao & Meighen, 1989), *Vibrio campbellii* AI-2 is a furanosyl borate diester, 3A-methyl-5,6-dihydro-furo [2,3-$_D$][1,3,2]dioxaborole-2,2,6,6 A tetraol (Chen et al., 2002), and *Vibrio campbellii* CAI-1 is a (Z)-3aminoundec-2-en-4-one (Ng et al., 2011).



HAI-1 AI-2 CAI-1

Figure 7. Structure of the *Vibrio campbellii* quorum sensing signal molecules

The autoinducers are detected at the cell surface by distinct membrane-bound, two-component receptor proteins that feed a shared phosphorylation/dephosphorylation signal transduction cascade controlling the production of the transcriptional regulator protein LuxR (Taga & Bassler, 2003). The concentration of LuxR depends on the concentration of five small regulatory RNAs, which is determined by the phosphorylation status of LuxO (Ng & Bassler, 2009). The phosphorylation status of LuxO in its turn is determined by the net result of the kinase and phosphatase activities of the three receptors and thus is dependent on the concentration of the three autoinducers (Defoirdt, 2013b).

Recently, a second master regulator, AphA, has been described (Rutherford et al., 2011). The levels of this second master regulator are inversely related to those of LuxR. Some quorum sensing target genes are controlled by one of the regulators, others are controlled by both of them. In total, hundreds of genes are controlled by these regulators. In addition to controlling the production of the master regulators, the small regulatory RNAs can regulate the expression of certain genes directly by binding to their mRNA (at least, in *V. cholerae*) (Hammer & Bassler 2007; Defoirdt, 2013b). The quorum sensing system of *Vibrio campbellii* is depicted in **Figure 8**.

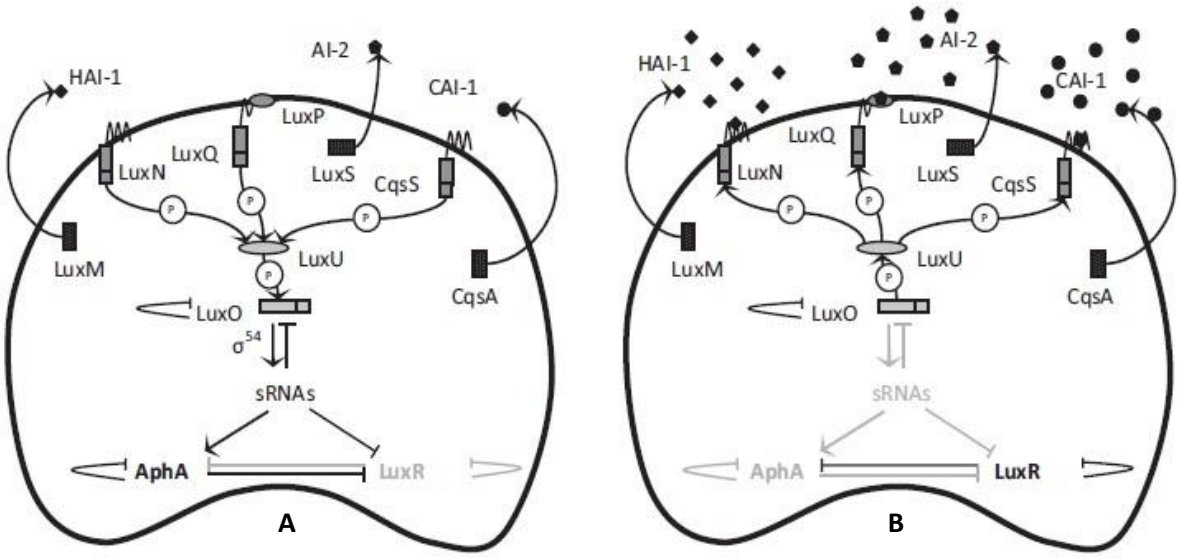


Figure 8. The multi-channel quorum sensing system in *Vibrio campbellii*. The LuxM, LuxS and CqsA enzymes synthesize the autoinducers HAI-1, AI-2 and CAI-1, respectively. These autoinducers are detected at the cell surface by the LuxN, LuxQ and CqsS two-component receptor proteins, respectively. Detection of AI-2 by LuxQ requires the periplasmic protein LuxP. (**A**) In the absence of autoinducers, the receptors autophosphorylate and transfer phosphate to LuxO via LuxU. Phosphorylation activates LuxO, which together with σ^{54} activates the production of five small regulatory RNAs (sRNAs). The sRNAs promote translation of the master regulator AphA and inhibit translation of the master regulator LuxR. (**B**) In the presence of high concentrations of the autoinducers, the receptor proteins switch from kinases to phosphatases, which results in dephosphorylation of LuxO. Dephosphorylated LuxO is inactive and therefore, the sRNAs are not formed, AphA is not translated and LuxR is translated. AphA and LuxR are transcriptional regulators that (either individually or together) affect the transcription of many target genes (Defoirdt, 2013b).

Some studies reported that quorum sensing has been known to regulate the expression of different virulence factors in pathogenic bacteria. Some virulence factors such as biofilm formation, exoprotease and siderophore production of aquaculture pathogens *Aeromonas hydrophila* and *Aeromonas salmonicida* has been shown to be regulated by quorum sensing and cause lethality in Burbot (*Lota lota* L.) larvae (Natrah et al., 2012; Swift et al., 1999). Motility, biofilm formation and type III secretion system are virulence factors regulated by quorum sensing in pathogenic *Edwardsiella tarda*, the causative agent of edwarsiellosis in Japanese flounder (*Paralichthys olivaceus*) and turbot *(Scolpthalmus maximus*) (Han et al., 2009; Leung et al., 2012).

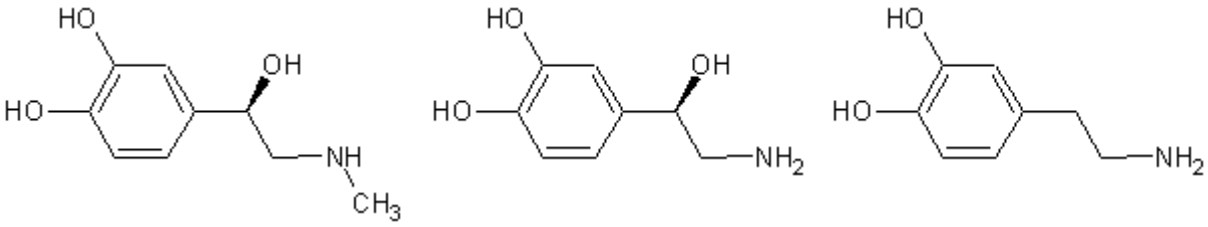
As the most important pathogen in aquaculture, many studies were dealing with quorum sensing-regulated virulence factors in Vibrios. For instance, in *Vibrio alginolyticus*, the virulence factors such as protease production, haemolytic activity, and extracellular polysaccharide production are regulated by quorum sensing and cause a high mortality in red sea bream (*Pagrus major*) (Rui et al., 2007; Wang et al., 2007; Ye et al., 2008). Quorum sensing also regulates the type III secretion system, the production of siderophore, chitinase, metalloprotease, phospholipase and extracellular polysaccharide in *Vibrio campbellii* lead to the lethality of brine shrimp *Artemia franciscana* and rotifer (*Brachionus plicatilis*) (Ruwandeepika et al., 2012). Other virulence factors such as motility, adhesion, cell-to-cell aggregation and biofilm formation in *Vibrio salmonicida* caused mortality in Atlantic salmon (Bjelland, 2012), are also known to be regulated by quorum sensing. Consequently, considerable research effort these days is directed towards the development of quorum sensing-disrupting techniques (Defoirdt et al., 2011a).

Host-pathogen signaling

Another important factor that also determines the expression of virulence factors is the presence and detection of host factors. The metabolic condition of the host (such as stress) and some metabolic products have a significant impact on the success of bacterial infection.

Catecholamine stress hormones

Host stress has been known for a long time to influence the outcome of host-microbe interactions, and this has been associated with a decreased activity of the host defense system (Verbrugghe et al., 2012). Recently, evidence has been provided that infectious bacteria have evolved specific detection systems for stress hormones produced by their host and that detection of these stress hormones results in increased virulence (Lyte, 2004). Most research in this respect has been carried out on the effects of the catecholamine hormones adrenaline (epinephrine), noradrenaline (norepinephrine) and dopamine (**Figure 9**) (Freestone et al., 2008).



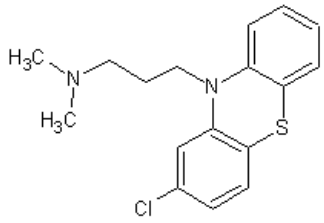
Adrenaline | Noradrenaline/ Norepinephrine | Dopamine

Figure 9. Structure of the catecholamine hormones

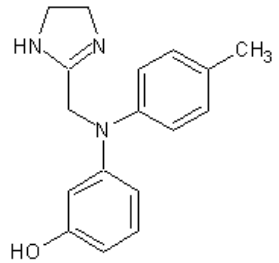
It has been reported that catecholamine stress hormones can influence growth, motility, biofilm formation, and/or virulence of intestinal pathogens such as *Escherichia coli* and *Salmonella* spp. (Verbrugghe et al., 2012) and interestingly, catecholamine can increase growth and type III secretion system in human pathogenic *Vibrio parahaemolyticus* in serum-based media (Nakano et al., 2007b). Serum is used in this kind of experiments to mimic the host environment where the availability of iron is limited by the high affinity ferric-iron-binding protein transferrin in serum (Freestone et al., 2008). The mechanistic explanation that prevails in literature is that catecholamines form complexes with transferrin, which reduces the ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}). This results in weakening of the bond between iron and transferrin, and therefore iron becomes available for the bacteria (Sharaff & Freestone, 2011; Freestone, 2013).

Catecholamines might also be important with respect to the virulence of aquaculture pathogenic bacteria as both aquatic invertebrates and vertebrates release these hormones into circulation system in response to stress (Ottaviani & Franceschi, 1996; Lacoste et al., 2001). Another study from Wang and co- workers (2011) also reported that catecholamine stimulates the flagellum motility, fimbriae and type III secretion system of the fish pathogen *Edwardsiella tarda*.

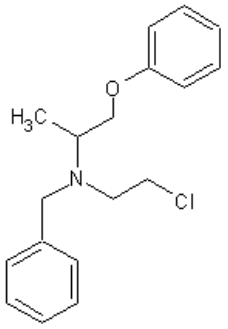
It has also been reported that antagonists of eukaryotic catecholamine receptors (**Figure 10**) were able to neutralize some of the effects of catecholamines on bacteria (Freestone et al., 2007). Another study showed that both α-adrenergic and β-adrenergic receptor antagonists were able to block the response of *E. coli* O157:H7 to norepinephrine and epinephrine (Sperandio et al., 2003).



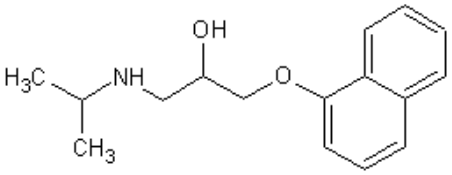
Chlorpromazine (dopaminergic)



Phentolamine (reversible α-adrenergic)



Phenoxybenzamine (irreversible α-adrenergic)



Propanolol (β-adrenergic)

Figure 10. Antagonists of eukaryotic catecholamine receptors

Mucin, bile salts and cholesterol

Apart from host stress hormones, virulence factor expression is also affected by other host factors, such as mucin, bile salts and cholesterol (Defoirdt, 2013, Li et al., 2014). Those three host factors has been shown to increase production of virulence factors (protease activity, flagellar motility, biofilm formation and exopolysaccharide production) in *Vibrio anguillarum* without any influence on growth of the bacterium. Moreover, pretreatment of *V. anguillarum* with these compounds increased its virulence towards gnotobiotic sea bass (*Dicentrarchus labrax*) larvae (Li et al., 2014). In addition, bile has also been reported to induce production of virulence factors including type III secretion system-related proteins, haemolysins, and capsular polysaccharide of *Vibrio parahaemolyticus* (Hsieh et al., 2003).

ANTIVIRULENCE THERAPY FOR AQUACULTURE

Some alternative methods have been developed in order to treat bacterial infection in aquaculture without using antibiotics (Defoirdt et al., 2011a), such as bacteriophage (Nakai & Park, 2002), inhibition of chromosome II replication in vibrios (Yamaichi et al., 2009), the growth inhibition of pathogenic bacteria by using short-chain fatty acids and polyhydroxyalkanoates (Vasquez at al., 2005; Defoirdt et al., 2006b) and inhibition of virulence gene expression as referred to antivirulence therapy (Clatworthy et al., 2007). In terms of antivirulence therapy, the inhibition of virulence factors can consist of either interfering with the regulation of virulence factor expression (thereby often affecting several virulence factor at once) or specifically inhibiting a certain virulence factor (Defoirdt, 2013b). With respect to aquaculture pathogens, quorum sensing inhibition is the only strategy that thus far has been tested (Defoirdt et al., 2007a; Brackman et al., 2008; Benneche et al., 2011; Defoirdt et al., 2012; Natrah et al., 2012).

The increasing knowledge of the regulation of bacterial pathogenesis by quorum sensing lead to a significant research effort on inhibiting these bacterial cell-to-cell communication mechanisms, called quorum quenching. Quorum sensing inhibition could be done by different mechanisms such as inhibition of signal molecule biosynthesis, application of

quorum sensing antagonists, chemical inactivation and enzymatic biodegradation of quorum sensing signal molecules (Defoirdt et al., 2004). Each of these mechanisms still needs to be explored further in order to find the best method to treat bacterial diseases in many different fields including aquaculture.

Inhibition of quorum sensing signal molecules biosynthesis has been done by application of substrate analogues (Defoirdt et al., 2004). For instance, S-adanosylcysteine (analogues of S-adenosylmethionine) has been found to inhibit the activity of the *Pseudomonas aeruginosa* AHL synthase RhlI by up to 97% (Parsek et al., 1999). S-adenosylmethionine is a substrate for homologues of the *V. fischeri* LuxI protein, that catalyse the biosysnthesis of AHL signal molecules (Whitehead et al., 2001). It might be possible to use the S-adenosylmethionine analogues as quorum sensing inhibitors, without affecting other vital processes in prokaryotic and eukaryotic organisms (Defoirdt et al., 2004).

Secondly, the detection of quorum sensing signal molecules can be blocked by application of quorum sensing antagonists. One class of quorum sensing antagonists is AHL molecules with a different side chain when compared to the natural AHLs produced by the specific bacterium of interest. It has been reported that long-chain AHLs can reduce virulence factor production in the aquaculture pathogens *Aeromonas hydrophila* and *Aeromonas salmonicida,* both of which naturally produce short-chain AHLs (Swift et al., 1999) and protect larvae of burbot (*Lota lota*) from infection by these pathogens (Natrah et al., 2012).

Another class of antagonists is quorum sensing-disrupting compounds or technically known as quorum sensing inhibitors. The most intensively studied class of quorum sensing inhibitors is halogenated furanones (**Figure 11A**), which have been shown to disrupt AHL-mediated quorum sensing by interacting with the LuxR-type AHL receptor, thereby reducing the amount of LuxR available for AHL as transcriptional regulator (Menefield et al., 2002). Interestingly, the furanones were also shown to block the multichannel quorum sensing systems of vibrios by decreasing the DNA-binding activity of the master regulator $LuxR_{vh}$ (not homologuous to *V. fischeri* LuxR) (Defoirdt et al., 2007b). These products have been

documented to protect both fish and crustaceans against vibriosis (Rasch et al., 2004; Defoirdt et al., 2006a). However, halogenated furanones are toxic to higher organisms in practical application, with toxic concentrations being only slightly higher than quorum sensing-disrupting concentrations (Defoirdt et al., 2006a). In addition to natural compounds produced by marine algae, the derivative of brominated furanone (**Figure 11B**) has also been synthesized (Janssens et al., 2008b).

Since the quorum sensing-disrupting furanones are toxic towards higher organisms, these compounds are considered as not safe for practical application in aquaculture. One study from Benneche and co-workers (2011) reported that the synthesis of brominated thiophenones, a sulphur analogues of brominated furanones, has similar effect on the quorum sensing system of vibrios, i.e. they decrease the DNA-binding activity of $LuxR_{vh.}$ The activity of brominated thiophenones and even more higher with respect to inhibition of biofim formation in *Staphylococcus epidermidis* (Lonn-Stensrud et al., 2011) and *Vibrio harveyi* (Benneche et al., 2011) than the corresponding furanones. The thiophenones concentration of 2,5 µM is having a similar effect to approximately 100 µM furanones (Defoirdt et al., 2012). Interestingly, one of thiophenone compound, (Z)-4-((5-(bromomethylene-2-oxo-2,5-dihydrothiphen-3-yl)-4-oxo-butanoic acid (**Figure 11C**) has a therapeutic index of 100 and hence a high potential to treat luminescent vibriosis (Defoirdt et al., 2012).

Another compound that has been known to have a quorum sensing-disrupting activity is cinnamaldeyhde (**Figure 11D**). Cinnamaldehyde is a non-toxic synthetic flavouring substance that is widely used in food, beverages, chewing gum, and the perfume and food chemistry, and is generally recognised as safe (Adams et al., 2004). This compound has been reported to have a similar activity as brominated furanones and thiophenones, namely to interfere with AI-2 based quorum sensing by decreasing DNA-binding activity of LuxR without inhibiting bacterial growth (Brackman et al., 2008). Furthermore, it has been found that cinnamaldehyde can inhibit biofilm formation in several *Vibrio* spp., can reduce the ability to survive starvation and antibiotic treatment and can reduce pigment and protease

production in *Vibrio anguillarum*. It also protected gnotobiotic *Artemia* shrimp against virulent *Vibrio harveyi* (Brackman et al., 2008) and blocked the virulence of both *Aeromonas hydrophila* and *Aeromonas salmonicida* towards burbot larvae (Natrah et al., 2012).

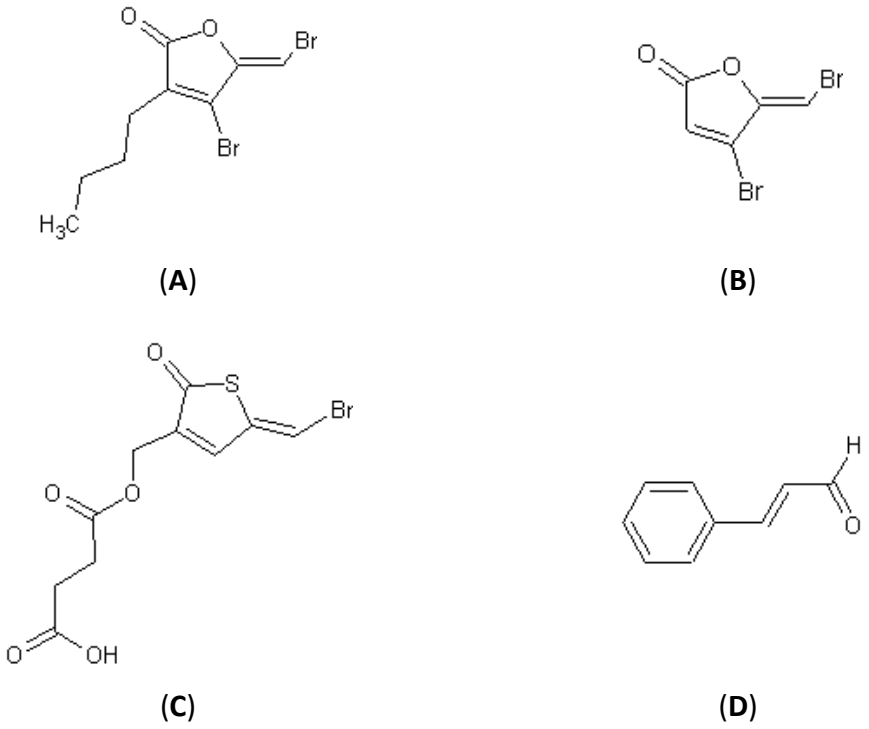


Figure 11. Structure of some quorum sensing-disrupting compounds. (**A**) The natural furanone (*5Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(*5H*)-furanone, produced by the red marine algae *Delisea pulchra*, (**B**) The synthetic derivative (*5Z*)-4-bromo-5-(bromomethylene)-2(*5H*)-furanone, (**C**) The brominated thiophenone (*Z*)-4-((5-bromomethylene)-2-oxo-2,5-dihydrothiophen-3-yl)-4-oxo-butanoic acid, and (**D**) Cinnamaldehyde (redrawn after Defoirdt, 2013b)

Quorum sensing can also be disrupted by enzymatic (bio)-degradation of signal molecules. This technique is one of the most intensively studied strategies for AHL quorum sensing disruption (Kalia & Purohit, 2011). The enzymatic degradation of AHL can be mediated by a lactonase, decarboxylase, acylase or deaminase (Hong et al., 2012) and haloperoxidase (Syrpas et al., 2014). The lactonase enzyme hydrolyzes and opens the ester bond of the lactone ring of AHLs, thereby inhibiting binding to the target transcriptional regulator (Dong et al., 2000). This enzyme, encoded by the AHL-inactivating activity (*aiiA*) gene, is

widespread in many *Bacillus* species (Dong et al., 2002). Lactonases hydrolyze both short chain and long chain AHLs with similar efficiency, but show no or little activity to other chemicals, including non-acyl lactones and aromatic carboxylic acid esters (Dong et al., 2007). Meanwhile, AHL acylases cleave the lactone ring, releasing a fatty acid and homoserine lactone (Fast & Tipton, 2012). Further, the fatty acid is used as a carbon source through β-oxidation pathway. The AHL cleaved by acylases enzyme cause significant reduction in effectiveness of the signaling molecule (Lin et al., 2003). However, acylases could only utilize AHLs with acyl side chain longer than eight carbons, as shown by AHL acylase enzyme PvdQ produced by *Pseudomonas aeruginosa*. Finally, haloperoxidases from *Delisea pulchra* and *Laminaria digitata* were shown to mediate the deactivation of β-keto-AHLs by electrophilic halogenation with bromine (Syrpas et al., 2014).

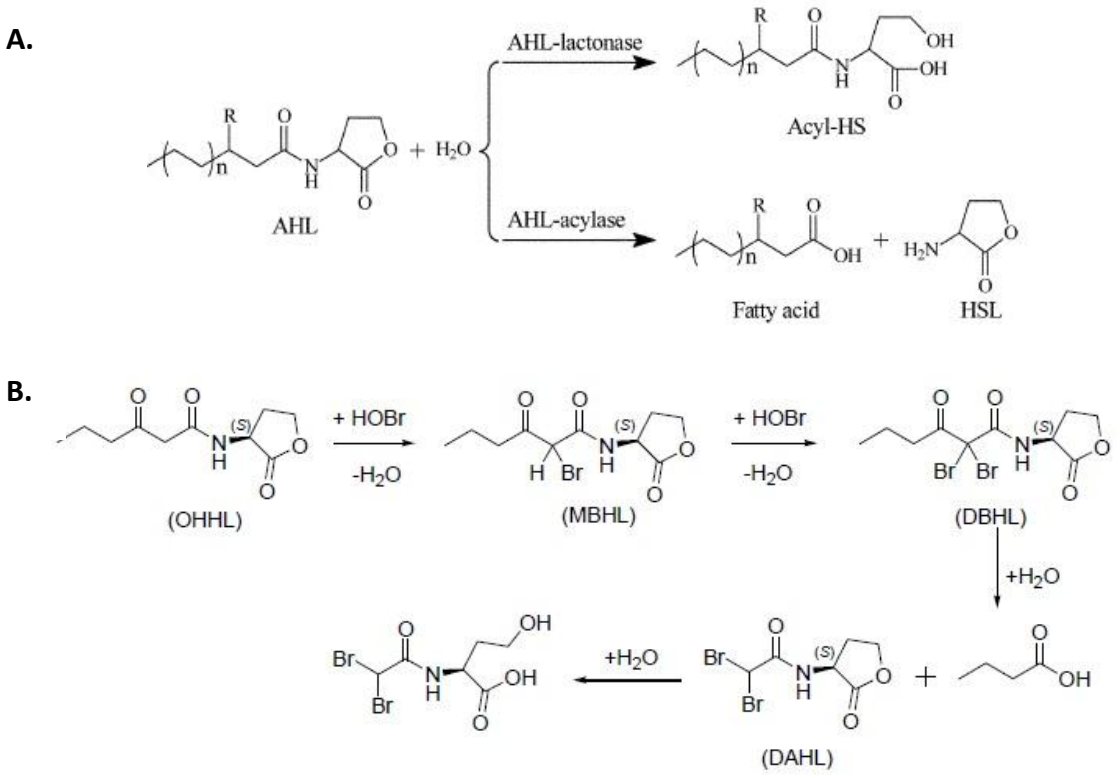


Figure 12. Enzymatic degradation of AHL molecules. (**A**)The degradation of AHL mediated by lactonase and acylase enzyme (Dong & Zhang, 2005), and (**B**) the degradation pathway of OHHL by haloperoxidases (Syrpas et al., 2014)

The biodegradation of AHL-signal molecules using AHL-degrading enrichment cultures has been shown to have a positive effect in some aquaculture animals (Tinh et al., 2007; Cam et al., 2009; Nhan et al., 2010). AHL-degrading enrichment cultures can be obtained by using media containing AHLs as the sole carbon and/or nitrogen source (Tinh et al., 2007), and pure strains of AHL-degrading *Bacillus* spp. have been isolated from these enrichment cultures (Defoirdt et al., 2011b). Hence, bacteria that are able to degrade quorum sensing signal molecules might be useful as a new kind of probionts for aquaculture.

CAN BACTERIA EVOLVE RESISTANCE TO QUORUM SENSING DISRUPTION?

In addition to mechanisms that render bacteria resistant to antibiotics (e.g. multidrug efflux pumps), Defoirdt at al., 2010 suggested that they may also evolve resistance to quorum sensing disruption or quorum quenching compounds based on the variation of the quorum sensing core genes between different strains in the same pathogen species. Since this variability is heritable, if it confers an advantage in fitness under quorum quenching treatment, that natural selection would favor the spread of quorum quenching resistance.

Many reports have shown that quorum sensing does not affect bacterial growth and therefore it was generally believed that quorum sensing disruption only has a small or even no effect on fitness. However, all the observations were made under conditions where bacteria were growing in nutrient-rich synthetic growth media (where quorum sensing-regulated genes are not essential for growth). Hence, the question that arises is whether quorum sensing disruption poses selective pressure on the bacteria where it really matters during infection (*in vivo*). If it does, then a mutant that is insensitive to quorum sensing disruption will have a selective advantage over the (sensitive) wild type and resistance will develop. However, at this moment, this kind of data is lacking.

RESEARCH GAPS

It has become clear that the wide and frequent use of antibiotics to control infections in aquaculture has resulted in the development and spread of antibiotic resistance. As this is

gradually rendering antibiotic treatments ineffective, new strategies to control bacterial infections are needed for a sustainable further development of the industry (Defoirdt et al., 2007a). Consequently, there is a need for scientific information on every aspect of the mechanisms by which the bacteria cause diseases, on how to treat the pathogens and on how this treatment will affect both the pathogens and their host. This information is crucial in order to be able to control bacterial diseases in the future. In order to obtain a better understanding of the possibilities for controlling bacterial infection in aquaculture by antivirulence therapy, this study was focused on the interference with pathogen-pathogen signaling (quorum sensing) and host-pathogen signaling.

Although quorum sensing had been reported to control the virulence of several aquaculture pathogens, not much information was available on the impact of quorum sensing and quorum sensing inhibition on commercially important species. The impact of quorum sensing inhibition on larviculture of the commercially important giant freshwater prawn *Macrobrachium rosenbergii* was investigated by different approaches such as the application of quorum sensing inhibitors (cinnamaldehyde, brominated furanones and brominated thiophenones). The possibility that novel quorum sensing signal molecule-degrading bacteria can be recruited from the aquaculture environment was also confirmed. Bacteria isolated from micro-algal cultures were able to produce quorum sensing inactivating enzymes and could protect the prawn larvae from disease.

In addition to quorum sensing, this study also focused on host-pathogen signaling through catecholamine stress hormones and its impact on the virulence of aquaculture pathogens. Recent literature suggested that sensing of catecholamines increased the virulence of enteric pathogens such as *E. coli* and *Salmonella* species. However, apart from these bacteria, little knowledge was available and the impact on the virulence of aquaculture pathogens was unknown. This study revealed that catecholamine stress hormones (dopamine and norepinephrine) increase the virulence of bacterial aquaculture pathogens and those antagonists of eukaryotic catecholamine receptors can block this effect.

RESEARCH OBJECTIVES AND THESIS OUTLINE

The general objective of this study was to investigate the impact of host-pathogen and pathogen-pathogen signaling on larviculture of giant freshwater prawn *M. rosenbergii*. All studies included *in vitro* experiments to confirm the effect of treatments on selected virulence factors, and *in vivo* experiments using *M. rosenbergii* larvae as a target species. The specific objectives and the thesis outline were as follows (**Figure 13)**:

CHAPTER 2 aimed at giving a literature overview of pathogenicity mechanisms in aquaculture pathogens and interference with these mechanisms (antivirulence therapy) as a sustainable alternative to antibiotics. This review summarizes problems related with antibiotic usage in aquaculture, the current knowledge of bacterial pathogenicity mechanisms and antivirulence therapy in aquaculture (focusing on host-pathogen and pathogen-pathogen signaling).

CHAPTER 3 aimed at investigating the impact of quorum sensing on the virulence of *Vibrio campbellii* towards *M. rosenbergii* and comparing this to the effect in brine shrimp (*Artemia franciscana*).

CHAPTER 4 and 5 aimed at examining the impact of interference with pathogen-pathogen signaling on vibriosis in *M. rosenbergii* larviculture. In **CHAPTER 4**, quorum sensing-disrupting compounds (cinnamaldehyde, brominated furanones and brominated thiophenone) were investigated. In **CHAPTER 5**, quorum sensing signal molecule-degrading bacteria isolated from microalgal cultures were used.

CHAPTER 6 aimed at studying the impact of the catecholamine stress hormones dopamine and norepinephrine on the virulence of the aquaculture pathogens *Vibrio campbellii* and *Vibrio anguillarum.*

CHAPTER 7 aimed at discussing the most important findings of this study and providing conclusions and suggestions for future research.

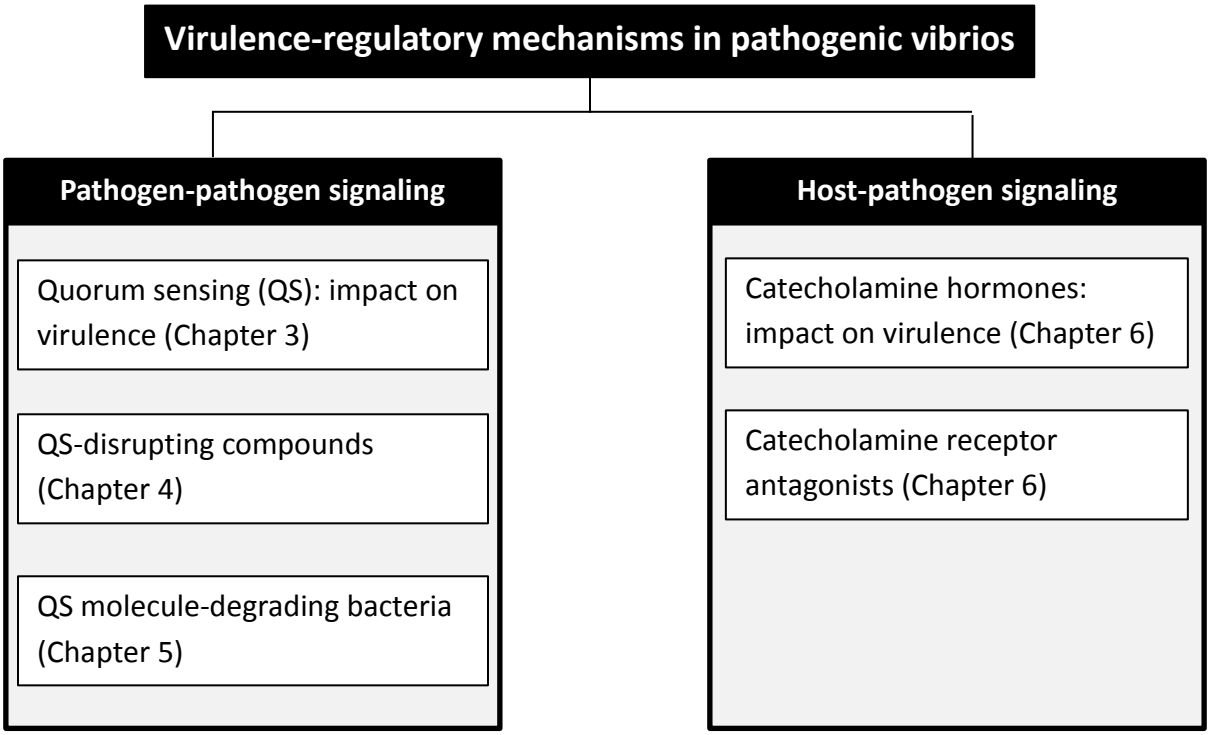


Figure 13. Schematic overview of the research objectives of this thesis.

CHAPTER 3

THE *VIBRIO CAMPBELLII* QUORUM SENSING SIGNALS HAVE A DIFFERENT IMPACT ON VIRULENCE OF THE BACTERIUM TOWARDS DIFFERENT CRUSTACEAN HOSTS

Redrafted after:
Pande GSJ, Natrah FMI, Sorgeloos P, Bossier P, Defoirdt T (2013) The *Vibrio campbellii* quorum sensing signals have a different impact on virulence of the bacterium towards different crustacean hosts. Veterinary Microbiology 167:540-545.

ABSTRACT

Pathogenic bacteria communicate with small signal molecules in a process called quorum sensing, and they often use different signal molecules to regulate virulence gene expression. *Vibrio campbellii*, one of the major pathogens of aquatic organisms, regulates virulence gene expression by a three channel quorum sensing system. Here we show that although they use a common signal transduction cascade, the signal molecules have a different impact on the virulence of the bacterium towards different hosts, i.e. the brine shrimp *Artemia franciscana* and the commercially important giant freshwater prawn *Macrobrachium rosenbergii*. These results suggest that the use of multiple types of signal molecules to regulate virulence gene expression is one of the features that allow bacteria to infect different hosts. Our findings emphasized that it is highly important to study the efficacy of quorum sensing inhibitors as novel biocontrol agents under conditions that are as close as possible to the clinical situation.

Key words: quorum sensing, vibriosis, host-microbe interaction, host-pathogen interaction

INTRODUCTION

Pathogenic bacteria are often capable of infecting multiple host species (Faruque et al., 1998; Salanoubat et al., 2002; He et al., 2004; Seshadri et al., 2006; Defoirdt et al., 2007a). This will require some level of flexibility in sensing of and responding to the environment since the bacteria will experience different environmental conditions in different hosts (especially when these are distantly related). One of the regulatory mechanisms involved in interacting with the environment is quorum sensing, a mechanism in which bacteria coordinate the expression of certain genes in response to the presence of small signal molecules (Hense et al., 2007). Quorum sensing systems have been reported to regulate the expression of virulence genes in many pathogenic bacteria, and many of them use different signal molecules to regulate virulence gene expression (Jayaraman & Wood, 2008; Ng & Bassler, 2009). However, it is often not clear why different signals are used, especially when they are controlling the same virulence factors and when they are using a common signal transduction cascade, as is the case in vibrios (Milton, 2006).

Vibrio campbellii BB120 (= ATCC BAA-1116; previously designated *Vibrio harveyi* (Lin et al., 2010)) and closely related bacteria are amongst the most important pathogens of aquatic organisms, causing significant losses in the aquaculture industry worldwide (Ruwandeepika et al., 2012). These pathogens can infect multiple hosts belonging to distantly related taxa, ranging from mollusks over crustaceans to fish (Defoirdt et al., 2007a). The species is also one of the model organisms in studies on quorum sensing in bacteria (Ng & Bassler, 2009). *Vibrio campbellii* BB120 contains a three-channel quorum sensing system, with three different types of signal molecules (HAI-1, AI-2 and CAI-1, respectively) feeding a common signal transduction cascade (**Figure 14**).

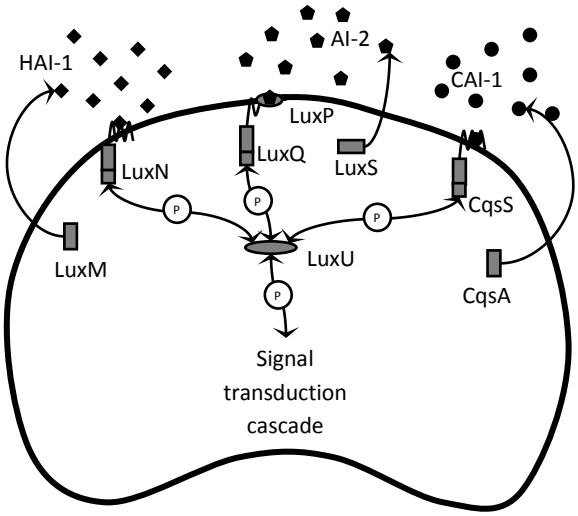


Figure 14. Quorum sensing in *Vibrio campbellii*. The LuxM, LuxS and CqsA enzymes synthesize the autoinducers HAI-1, AI-2 and CAI-1, respectively. These autoinducers are detected at the cell surface by the LuxN, LuxQ and CqsS two-component receptor proteins, respectively. Detection of AI-2 by LuxQ requires the periplasmic protein LuxP. The receptors feed a common phosphorylation/dephosphorylation signal tranducsction cascade regulating the expression of target genes. "P" denotes phosphotransfer

HAI-1, harveyi autoinducer-1, is 3-hydroxybutanoyl-L-homoserine lactone; AI-2, autoinducer-2, is the furanosyl borate diester 3A-methyl-5,6-dihydro-furo(2,3-D)(1,3,2)diox-aborole-2,2,6,6A-tetraol; and CAI-1, cholerae autoinducer-1, is (Z)-3-aminoundec-2-en-4-one (Ng & Bassler, 2009; Ng et al., 2011). *Vibrio campbellii* quorum sensing has been documented to control the expression of different virulence genes, including *vhp* metalloprotease (Mok *et* al., 2003; Ruwandeepika et al., 2011), a siderophore (Lilley & Bassler, 2004), type III secretion system components (Henke & Bassler, 2004a), chitinase A (Defoirdt et al., 2010b) and three phospholipase genes (Natrah et al., 2011b). We previously reported that AI-2 and CAI-1, but not HAI-1, are needed for full virulence of this bacterium towards brine shrimp larvae (Defoirdt et al., 2005; Defoirdt & Sorgeloos, 2012). Here, we report that HAI-1 and AI-2, but not CAI-1, are needed for full virulence towards the commercially important giant freshwater prawn *Macrobrachium rosenbergii*. These data suggest that the use of different types of signal molecules to regulate virulence gene expression is one of the features that allow bacteria to infect different hosts.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Vibrio campbellii strains used in this study are described in **Table 4**. All strains were stored at -80^{o}C in 40% glycerol and the stocks were streaked onto Luria-Bertani agar containing 12 g.l^{-1}Instant Ocean synthetic sea salt (Aquarium Systems Inc., Sarrebourg, France) (LB_{12}) or 35 g.l^{-1} Instant Ocean (LB_{35}) for use in giant freshwater prawn and brine shrimp, respectively. After 24 hours of incubation at 28^{o}C, a single colony was inoculated into 5 ml fresh LB broth with appropriate salinity and incubated overnight at 28^{o}C under constant agitation (100 min^{-1}).

Table 4. *Vibrio campbellii* strains used in this study.

Strain	Relevant information	Reference
BB120	Wild type from which all other strains are derived	Bassler et al. (1997)
BB120RifR	Spontaneous rifampicin resistant mutant of BB120	This study
BB152	Mutation in *luxM* (HAI-1 synthase)	Bassler et al. (1994)
MM30	Mutation in *luxS* (AI-2 synthase)	Surette et al. (1999)
MM77	Mutation in *luxM* and *luxS*	Surette et al. (1999)
MM77RifR	Spontaneous rifampicin resistant mutant of MM77	This study
JMH603	Mutation in *cqsA* (CAI-1 synthase)	Henke & Bassler (2004b)

Selection of rifampicin resistant mutants of *Vibrio campbellii* BB120 and MM77

100 µl of densely grown cultures of BB120 and MM77 (OD_{600} of 1) were inoculated into tubes containing 5 ml of fresh LB_{12} broth supplemented with 50 mg.l^{-1} rifampicin (Sigma)

and incubated for 5 days at 28^{o}C under constant agitation (100 min^{-1}). The grown cultures were inoculated into fresh LB_{12} broth with 50 mg.l^{-1} rifampicin and incubated overnight. The grown cultures were stored at -80^{o}C in 40% glycerol until use.

Signal molecules

HAI-1, N-3-hydroxybutanoyl-L-homoserine lactone (Sigma) was dissolved in distilled water at 1000 mg.l^{-1}. AI-2 precursor (S)-4,5-dihydroxy-2,3-pentadione (DPD) was obtained from OMM Scientific Inc. (Dallas, Texas, USA). 1 mg.l^{-1} of corresponding signal molecule was added to the rearing water (Natrah, 2011).

Axenic hatching of brine shrimp

Decapsulation and hatching of axenic brine shrimp was performed as described previously (Defoirdt et al., 2005), with some modifications. Briefly, 200 mg cysts of *Artemia fransiscana* (Ocean Nutrition Europe, Essen, Belgium) from Great Salt Lake were hydrated in a 50 ml tube containing 18 ml distilled water for 1 h. Sterile cysts were obtained via decapsulation using 660 µl NaOH (32%) and 10 ml NaOCl (50%). The reaction was stopped after 2 minutes by adding 14 ml $Na_2S_2O_3$ (10 g.l^{-1}). The decapsulated cysts were washed with fresh autoclaved synthetic sea water (35 g.l^{-1} Instant Ocean) over a 100 µm sieve and transferred to two sterile 50 ml tubes, each containing 30 ml sterile synthetic sea water. The tubes with decapsulated cysts were incubated at 28^{o}C for 24 hours on a rotor under constant light. For brine shrimp larvae to be used as feed for giant freshwater prawn larvae, brackish water (12 g.l^{-1} Instant Ocean) was used.

Brine shrimp challenge test

The brine shrimp challenge test was performed as described previously (Defoirdt et al., 2006a). Briefly, the shrimp were cultured in groups of 20 larvae in glass tubes containing 10 ml synthetic sea water (35 g.l^{-1} Instant Ocean). The larvae were fed an autoclaved suspension of *Aeromonas* sp. LVS3 bacteria at 10^7 cells.ml^{-1} and *Vibrio campbellii* strains were added at 10^5 CFU.ml^{-1}.

Giant freshwater prawn challenge tests

Two different prawn broodstocks were maintained at our laboratory (further denoted broodstock 1 and 2, respectively). Prawn broodstock maintenance was performed according to Cavalli et al. (2001) and water quality parameters were adjusted according to New (2003). The larvae were obtained from a single breeder. A matured female which had just completed its pre-mating moult was mated with a hard-shelled male as described before (Baruah et al., 2009). The female with fertilized eggs was then maintained for 20 to 25 days to undergo embryonic development. When the eggs were fully ripe (indicated by dark grey color), the female was transferred to a hatching tank (30 l) containing brackish water (6 $g.l^{-1}$ Instant Ocean). The water temperature was maintained at 28°C by a thermostat heater. The newly hatched larvae with yolk were left for 24 hours in the hatching tank. The next day, prawn larvae with absorbed yolk were distributed in groups of 25 larvae in 200 ml glass cones containing 100 ml fresh autoclaved brackish water (12 $g.l^{-1}$ Instant Ocean). The glass cones were placed in a water bath maintained at 28°C and was provided with aeration. The larvae were fed daily with 5 brine shrimp larvae/prawn larva and acclimatized to the experimental conditions for 24 hours. During the experiments, water quality parameters were kept at minimum 5 $mg.l^{-1}$ dissolved oxygen, maximum 0.5 $mg.l^{-1}$ ammonium-N and maximum 0.05 $mg.l^{-1}$ nitrite-N. Prawn larvae were challenged with *Vibrio campbellii* by adding the strains at 10^6 $CFU.ml^{-1}$ to the culture water on the day after first feeding. Signal molecules were also added to the culture water on the day after first feeding. Survival was counted daily in the treatment challenged to wild type *Vibrio campbellii* and the challenge test was stopped when more than 50% mortality was achieved in this treatment (in order to have enough larvae remaining for the growth measurement). At this time point, larval survival was determined in all treatments by considering that only those larvae presenting movement of appendages were alive. The growth parameter larval stage index (LSI) was determined according to Maddox & Manzi (1976) on 5 randomly sampled larvae from each cone and calculated as: $LSI = \Sigma Si/N$, which Si : stage of the larva (i = 1 to 12) and N : the number of larvae examined.

Determination of *Vibrio campbellii* levels in the prawn larval gut and the culture water

In the challenge test using the rifampicin resistant mutants, larvae and water samples were collected twice, on day 5 and day 8. Three samples of 10 larvae were taken at random and were collected on sterile 500 µm sieves. The animals were anaesthetized in a 0.1% benzocaine solution (Sigma) for 10 seconds. Surface bacteria were removed according to Nhan et al. (2010) by immersing the samples in a 0.1% benzalkonium chloride solution (Sigma) for 10 seconds and then rinsing them three times with sterile brackish water. The larvae were then transferred into sterile plastic bags containing 4 ml of brackish water and homogenized for 60 seconds using a stomacher machine, as described by Nhan et al. (2010). Subsequently, the samples were serially diluted, plated on LB_{12} agar containing 100 $mg.l^{-1}$ rifampicin and incubated for 48 hours at $28^{o}C$.

Statistical data analysis

Statistical analyses were performed using the SPSS software, version 20. The giant freshwater prawn survival data were arcsin transformed in order to satisfy normal distribution and homoscedasticity requirements.

RESULTS

The impact of *Vibrio campbellii* quorum sensing on survival of brine shrimp larvae

In a first experiment, we challenged brine shrimp larvae with *Vibrio campbellii* wild type strain BB120 and different quorum sensing mutants defective for signal molecule production. Consistent with our previous reports (Defoirdt et al., 2005; Defoirdt & Sorgeloos, 2012), the AI-2 and CAI-1 deficient mutants were significantly less virulent than the wild type, whereas the HAI-1 deficient mutant was as virulent as the wild type (**Figure 15A**).

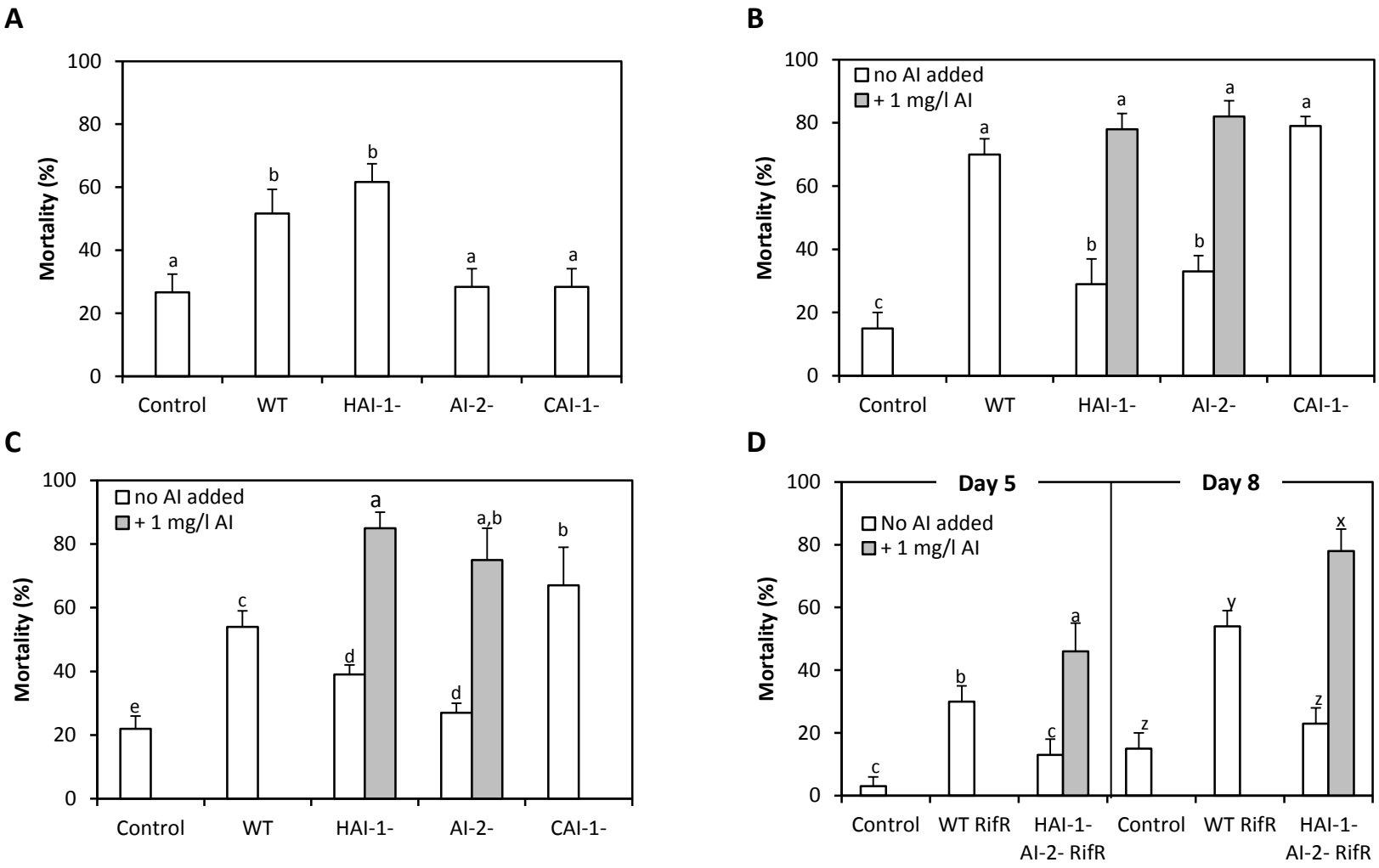

A
Mortality (%)
100
80
60
40
20
0
a
b
b
a
a
Control
WT
HAI-1-
AI-2-
CAI-1-
B
Mortality (%)
no AI added
+ 1 mg/l AI
c
a
b
a
b
a
a
Control
WT
HAI-1-
AI-2-
CAI-1-
C
Mortality (%)
no AI added
+ 1 mg/l AI
e
c
d
a
d
a,b
b
Control
WT
HAI-1-
AI-2-
CAI-1-
D
Day 5
Day 8
Mortality (%)
No AI added
+ 1 mg/l AI
c
b
c
a
z
y
z
x
Control
WT RifR
HAI-1-
AI-2- RifR
Control
WT RifR
HAI-1-
AI-2- RifR

Figure 15. Impact of the three quorum sensing signals on virulence of *Vibrio campbellii* towards two different hosts. **(A)** Percentage mortality of brine shrimp larvae after 2 days of challenge with *Vibrio campbellii* wild type and signal molecule synthase mutants. Error bars represent the standard deviation of three replicate brine shrimp cultures. **(B)** Percentage mortality of giant freshwater prawn larvae originating from broodstock 1 after 5 days of challenge with *Vibrio campbellii* wild type and signal molecule synthase mutants. The HAI-1- and the AI-2- negative mutant were tested without and with (grey bars) 1 $mg.l^{-1}$ of the corresponding signal. Error bars represent the standard deviation of five replicate prawn cultures. **(C)** Percentage mortality of giant freshwater prawn larvae originating from broodstock 2 after 8 days of challenge with *Vibrio campbellii* wild type and signal molecule synthase mutants. The HAI-1- and the AI-2- negative mutant were tested without and with (grey bars) 1 $mg.l^{-1}$ of the corresponding signal. Error bars represent the standard deviation of five replicate prawn cultures. **(D)** Percentage mortality of giant freshwater prawn larvae originating from broodstock 2 after 8 days of challenge with rifampicin resistant mutants of *Vibrio campbellii* wild type and the HAI-1 AI-2 synthase double mutant MM77 (without and with 1 $mg.l^{-1}$ HAI-1 and 1 $mg.l^{-1}$ AI-2). Error bars represent the standard deviation of five replicate prawn cultures.

The brine shrimp challenge test was performed by T. Defoirdt. For each panel, bars with a different letter are significantly different from each other (ANOVA with Tukey's post-hoc test; $p < 0.05$). "Control" refers to unchallenged animals that were otherwise treated in the same way as the challenged animals.

The impact of *Vibrio campbellii* quorum sensing on survival and growth of giant freshwater prawn larvae

In further experiments, we challenged giant freshwater prawn larvae to the *Vibrio campbellii* wild type and the signal molecule deficient mutants. The wild type BB120 was found to be pathogenic to the prawn larvae as it caused significant mortality to larvae originating from both broodstocks maintained in our laboratory. The mortality of larvae originating from broodstock 1 that were challenged to the wild type reached the 50% level after 5 days of challenge (**Figure 15B**). The larvae of broodstock 2 were stronger than those originating from broodstock 1 as it took 8 days of challenge with the wild type strain to reach the 50% mortality level (**Figure 15C**). Further, the HAI-1 and AI-2 deficient mutants showed a significantly decreased virulence to larvae from both broodstocks (**Figure 15B and 15C**). Addition of the signal molecules together with the synthase mutants restored the virulence of the mutants (**Figure 15B and 15C**), whereas the signal molecules had no effect on the survival of non-challenged larvae (survival was 72 ± 3 % and 74 ± 14 % for HAI-1 and AI-2, respectively, versus 60 ± 11 % in untreated larvae). In contrast to the HAI-1 and AI-2 deficient mutants, significant mortality (similar to that caused by the wild type) was observed in prawn larvae challenged with the CAI-1 deficient mutant (**Figure 15B and 15C**). Finally, none of the strains affected growth of the surviving larvae (data not shown).

Impact of quorum sensing on *Vibrio campbellii* density in the giant freshwater prawn larval gut and culture water

In order to be able to distinguish *Vibrio campbellii* strains added to the prawn cultures from the natural microbiota associated with the cultures, we used rifampicin resistant mutants of the wild type and the HAI-1 and AI-2 synthase double mutant MM77. These strains were used to challenge larvae originating from broodstock 2 and showed a similar virulence as observed for their non-resistant counterparts. Mortality of larvae challenged to the wild

type reached the 50% level after 8 days of challenge and was significantly higher than that of larvae challenged to the HAI-1 and AI-2 deficient mutant. Addition of the signal molecules together with the double mutant restored its virulence (**Figure 15D**). Although the *Vibrio campbellii* density showed a gradual increase as the challenge period progressed, the levels inside the larval gut or culture water were not significantly different between the wild type and the HAI-1 and AI-2 double mutant MM77 (**Table 5**).

Table 5. *Vibrio campbellii* density in the gut and culture water of giant freshwater prawn larvae after five and eight days of challenge (average ± standard deviation of three replicates plate counts on LB_{12} agar with 100 $mg.l^{-1}$ rifampicin). Treatments correspond to those in **Figure 15D**.

Treatment	**Gut (log CFU/larva)**		**Culture water (log CFU/ml)**	
	Day 5	**Day 8**	**Day 5**	**Day 8**
Control	ND	ND	ND	ND
WT RifR	3.9 ± 0.2	4.8 ± 0.2	5.7 ± 0.1	6.1 ± 0.0
HAI-1$^-$ AI-2$^-$ RifR	3.8 ± 0.2	4.7 ± 0.2	5.6 ± 0.1	6.1 ± 0.0
HAI-1$^-$ AI-2$^-$ RifR + 1 $mg.l^{-1}$ HAI-1 + 1 $mg.l^{-1}$ AI-2	4.0 ± 0.1	4.9 ± 0.2	5.8 ± 0.1	6.2 ± 0.0

ND : Not Detected

DISCUSSION

Vibrio campbellii, one of the major pathogens of aquatic animals, uses a three-channel quorum sensing system to regulate virulence gene expression (Defoirdt et al., 2007a; Ruwandeepika et al., 2012). The data presented in this paper show that although they feed a common signal transduction cascade, the three different quorum sensing signal molecules produced by the bacterium have a different impact on its virulence towards different crustacean hosts. AI-2 and CAI-1 are needed for full virulence of the pathogen towards brine

shrimp larvae, whereas HAI-1 has no effect. In contrast, HAI-1 and AI-2 are needed for full virulence towards giant freshwater prawn larvae, whereas CAI-1 has no effect in this host. We propose three different mechanisms that either alone or together might explain the apparent discrepancy between the fact that the receptors of the three signal molecules have been reported to feed a common signal transduction cascade and the fact that the signals have a different impact on virulence towards different hosts. First, the molecules can have a different stability in different environments (and thus in different hosts). For instance, short side chain acylhomoserine lactones (such as *Vibrio campbellii* HAI-1) have been reported to have low stability at pH values above 8 (Byers et al., 2002; Yates et al., 2002). Alkaline hydrolysis of the lactone ring of the HAI-1 signal might explain its inactivity in the brine shrimp host which is cultured in sea water (pH ≥ 8). Another possibility might be that the hosts produce enzymes that can inactivate (one of) the signal molecules. Acylhomoserine lactone inactivating enzyme activity for instance, has been reported in different higher organisms (Dong et al., 2007). A second possible explanation is that the expression of signal molecule synthases and/or receptors might be different in different environments. Indeed, Teng et al. (2011) recently reported that *Vibrio campbellii* can alter the ratio between the signal molecule receptors, allowing cells to pay more attention to one of the signals under certain conditions. A third possibility is that there might be not yet identified signal transduction cascades that are not affected by all three signals. We recently reported that the expression of a *Vibrio campbellii* hemolysin gene (*vhh*) is decreased in an AI-2 deficient mutant and an AI-2 receptor (*luxP*) mutant, whereas it is not in a mutant in which the known signal transduction cascade is inactivated (Ruwandeepika et al., 2011). This suggests that there might be an additional signal transduction cascade that is only affected by AI-2.

Many pathogenic bacteria use different quorum sensing signal molecules to regulate virulence gene expression (Jayaraman & Wood, 2008; Ng & Bassler, 2009). However, it is often not clear why different signals are used, especially when they are using a common

signal transduction cascade (as is the case in vibrios). Our data suggest that the use of different types of signal molecules might be one of the features that enable *Vibrio campbellii* to infect different host organisms. Indeed, if the bacterium would rely only on HAI-1 signalling, then it would fail to infect brine shrimp, whereas if it would rely only on CAI-1 signalling, then it would fail to infect giant freshwater prawn. Similar mechanisms might apply to other pathogens using multiple types of signal molecules to regulate virulence gene expression and specifically other *Vibrio* species, including animal and human pathogens such as *Vibrio alginolyticus* and *Vibrio parahaemolyticus* (both of which also belong to the *Harveyi* clade of vibrios), *Vibrio cholerae* and *Vibrio vulnificus.*

Because quorum sensing systems regulate the expression of virulence genes in many bacteria that are pathogenic to plants, animals and/or humans, quite some research effort currently is being directed towards the disruption of quorum sensing as a novel biocontrol strategy (Rasmussen & Givskov, 2006; Janssens et al., 2008a; Njoroge & Sperandio, 2009; Pan & Ren, 2009; Galloway et al., 2012; Kalia, 2013).

ACKNOWLEDGEMENTS

We thank Bonnie Bassler for providing us with the *V. campbellii* wild type and quorum sensing mutants. This work was funded by the Research Foundation of Flanders (FWO-Vlaanderen; project n^{o} 1.5.013.12N) and by the Directorate General of Higher Education of Indonesia through a doctoral scholarship to Pande Gde Sasmita Julyantoro. Tom Defoirdt is a postdoctoral fellow of FWO-Vlaanderen.

CHAPTER 4

QUORUM SENSING-DISRUPTING COMPOUNDS PROTECT LARVAE OF THE GIANT FRESHWATER PRAWN *MACROBRACHIUM ROSENBERGII* FROM *VIBRIO CAMPBELLII* INFECTION

Redrafted after
Pande GSJ, Scheie AA, Benneche T, Wille M, Sorgeloos P, Bossier P and Defoirdt T (2013) Quorum sensing-disrupting compounds protect larvae of the giant freshwater prawn *Macrobrachium rosenbergii* from *Vibrio harveyi* infection. Aquaculture 406-407:121-124

ABSTRACT

Vibriosis outbreaks caused by *Vibrio campbellii* and related species are amongst the major obstacles for the further expansion of giant freshwater prawn (*Macrobrachium rosenbergii*) larviculture. *Vibrio campbellii* regulates virulence gene expression through quorum sensing, bacterial cell-to-cell communication, and consequently, quorum sensing disruption has been suggested as an alternative strategy to control infections caused by these bacteria. Previous studies have shown that quorum sensing-disrupting compounds are able to disrupt quorum sensing in *Vibrio campbellii*. In this study, we demonstrated that the quorum sensing-disrupting compounds cinnamaldehyde, (*Z*-)-4-bromo-5-(bromomethylene)-2(5*H*)-furanone and (*Z*)-4-((5-(bromomethylene)-2-oxo-2,5-dihydrothiophen-3-yl)metoxy)-4-oxobutanoic acid increased the survival of giant freshwater prawn larvae when challenged to pathogenic *Vibrio campbellii*. Our *in vivo* challenge test showed that cinnamaldehyde and the thiophenone can protect the larvae from *Vibrio campbellii* infection when dosed to the culture water at 1 µM and 10 µM, whereas the brominated furanone offered protection at 1 µM but resulted in complete mortality at 10 µM. Although there were significant differences in survival between challenged larvae with and without addition of quorum sensing-disrupting compounds, there were no differences in growth (as determined by the larval stage index).

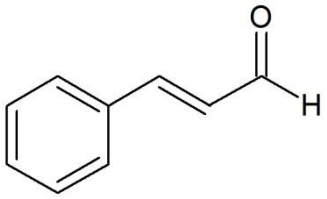
Keywords: Quorum quenching; Cinnamaldehyde; Furanone; Thiophenone; Virulence; Infection

INTRODUCTION

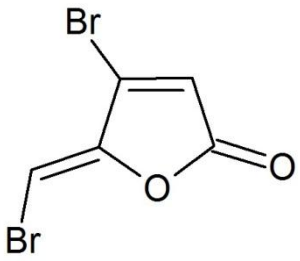
The giant freshwater prawn, *Macrobrachium rosenbergii* (de Man, 1879), is an important freshwater crustaceans from a commercial point of view. Freshwater prawn farming has expanded enormously since 1980, and in 2009 the global production reached a level of up to 80 times the production reported in 1980 (New & Nair, 2012). However, disease outbreaks are amongst the major obstacles to produce healthy and high quality seed for the further expansion of giant freshwater prawn culture (Nhan et al., 2010). Some studies have shown that *Vibrio* spp. are a major cause of disease (Tonguthai, 1997; Kennedy et al., 2006; New et al., 2010). In general, vibriosis is prevalent in the early life stages (eggs, larvae, and postlarvae) of giant freshwater prawn (Bhat & Singh, 1999), all of which need brackish water to survive (New et al., 2010). One of the species that has been isolated from affected giant freshwater prawn larvae is the luminescent bacterium *Vibrio campbellii* (Tonguthai, 1997; Bhat & Singh, 1999). The *Vibrio campbellii* BB120 (= ATCC BAA-1116) previously was designated as *Vibrio harveyi* (Lin et al., 2010). This species belongs to the *Harveyi* clade vibrios which are amongst the most important bacterial pathogens of aquatic animals, and cause significant losses in the aquaculture industry worldwide (Austin & Zhang, 2006; Ruwandeepika et al., 2012).

The frequent use of antibiotics to control vibriosis in hatcheries has led to the development and spread of antibiotic-resistant bacteria (Karunasagar et al., 1994; Moriarty, 1998), and alternative methods are needed to control these bacterial infections. Recently, it has become clear that the virulence of *V. campbellii* is under control of quorum sensing, a regulatory mechanism based on secreting and sensing small signal molecules called autoinducers (Cao & Meighen, 1989; Chen et al., 2002; Henke & Basler, 2004; Defoirdt et al. 2008; Natrah et al., 2011b). We previously reported that in *V. campbellii* quorum sensing regulates its virulence towards giant freshwater prawn larvae (Pande et al., 2013a). Consequently, the application of quorum sensing-disrupting agents might be a valid strategy to control vibriosis in this species. Quorum sensing-disrupting compounds such as

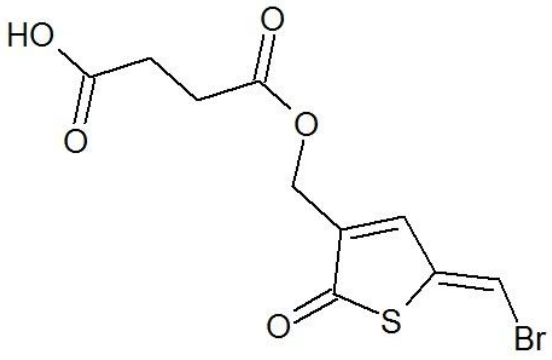
cinnamaldehyde, brominated furanones and brominated thiophenones (**Figure 16**) have been reported before to protect brine shrimp larvae from *V. campbellii* (Defoirdt et al., 2007b; Brackman et al., 2008; Defoirdt et al., 2012). In this study, we aimed at investigating the impact of these compounds on the survival and growth of giant freshwater prawn larvae when challenged to pathogenic *V. campbellii*.



Cinnamaldehyde



(*Z*-)-4-bromo-5-(bromomethylene)-2(5*H*)-furanone



(*Z*)-4-((5-(bromomethylene)-2-oxo-2,5-dihydrothiophen-3-yl)methoxy)-4-oxobutanoic acid

Figure 16. Structure of the quorum sensing-disrupting agents used in this study

MATERIALS AND METHODS

Bacterial strains and growth conditions

In this study we used *Vibrio campbellii* BB120 (=ATCC BAA-1116). The bacterium was stored at -80°C in 40% glycerol and the stocks were streaked onto Luria-Bertani agar containing 12 $g.l^{-1}$ synthetic sea salt (LB_{12}). After 24 hours of incubation at 28°C, a single colony was inoculated into 5 ml LB_{12} broth and incubated overnight at 28°C under constant agitation (100 min^{-1}). Bacterial density was measured spectrophotometrically at 600 nm.

Quorum sensing-disrupting compounds

Cinnamaldehyde (Sigma) was dissolved in distilled water at 1 mM and the brominated furanone (*Z*-)-4-bromo-5-(bromomethylene)-2(5*H*)-furanone (Sigma) was dissolved in methanol at 100 µM. The brominated thiophenone (*Z*)-4-((5-(bromomethylene)-2-oxo-2,5-dihydrothiopen-3-yl)methoxy)-4-oxobutanoic acid was synthesized as described before (Defoirdt et al., 2012) and dissolved in ethanol at 10 mM. All compounds were stored at -20°C. Control experiments were performed to verify that the solvents had no effect at the concentrations used.

Preparation of axenic *Artemia* nauplii as feed and giant freshwater prawn challenge test

The material and methods for preparations of axenic *Artemia* nauplii as feed for prawn larvae and the giant freshwater prawn larvae challenge test were performed similarly as described in **Chapter 3**.

Statistical data analysis

Statistical analyses were performed using the SPSS software, version 20. The larval survival data were arcsin transformed in order to satisfy normal distribution and homoscedasticity requirements. Data were analysed by one way ANOVA, followed by Tukey multiple range tests with a significance level set at 0.05.

RESULTS AND DISCUSSION

In this study we investigated the impact of the addition of three different quorum sensing-disrupting compounds, including cinnamaldehyde, the brominated furanone (*Z*-)-4-bromo-5-(bromomethylene)-2(5*H*)-furanone, and the thiophenone (*Z*)-4-((5-(bromomethylene)-2-oxo-2,5-dihydrothiophen-3-yl)-4-oxobutanoic acid) on the virulence of *V. campbellii* towards giant freshwater prawn larvae. *In vivo* challenge experiments revealed that the survival of challenged larvae significantly increased when these compounds were added to the culture water at 1 µM, offering a complete protection (no significant difference in survival when compared to non-challenged larvae) (**Figure 17**).

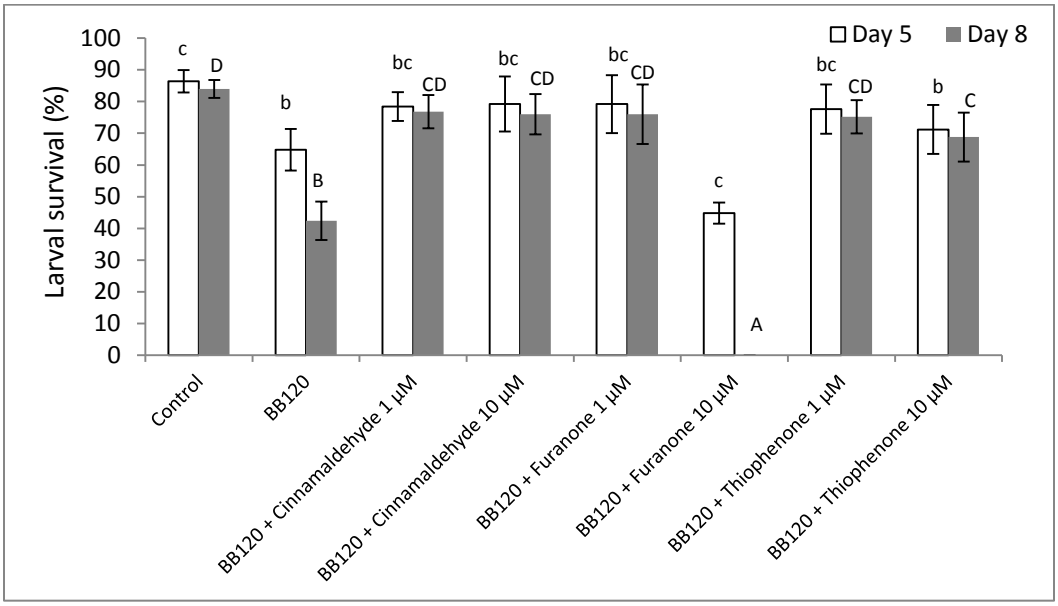


Figure 17. Percentage survival of giant freshwater prawn larvae after 5 and 8 days of challenge with *Vibrio campbellii* BB120, with and without quorum sensing-disrupting compounds (average ± standard deviation of five replicates). Bars with different letter are significantly different from each other (p<0.05). Cinnamaldehyde, the furanone (*Z*-)-4-bromo-5-(bromomethylene)-2(5*H*)-furanone, and the thiophenone (*Z*)-4-((5-(bromomethylene)-2-oxo-2,5-dihydrothiophen-3-yl)methoxy)-4-oxobutanoic acid were added to the culture water at the start of the experiment at 1 and 10 µM.

Increasing the concentration of the compounds to 10 µM did not further increase the survival of the larvae and 10 µM of furanone even resulted in complete mortality of the larvae. Importantly, the quorum sensing-disrupting compounds showed no negative impact on the growth of surviving prawn larvae since no difference in larval stage index (LSI) was observed between treated and untreated larvae (**Figure 18**).

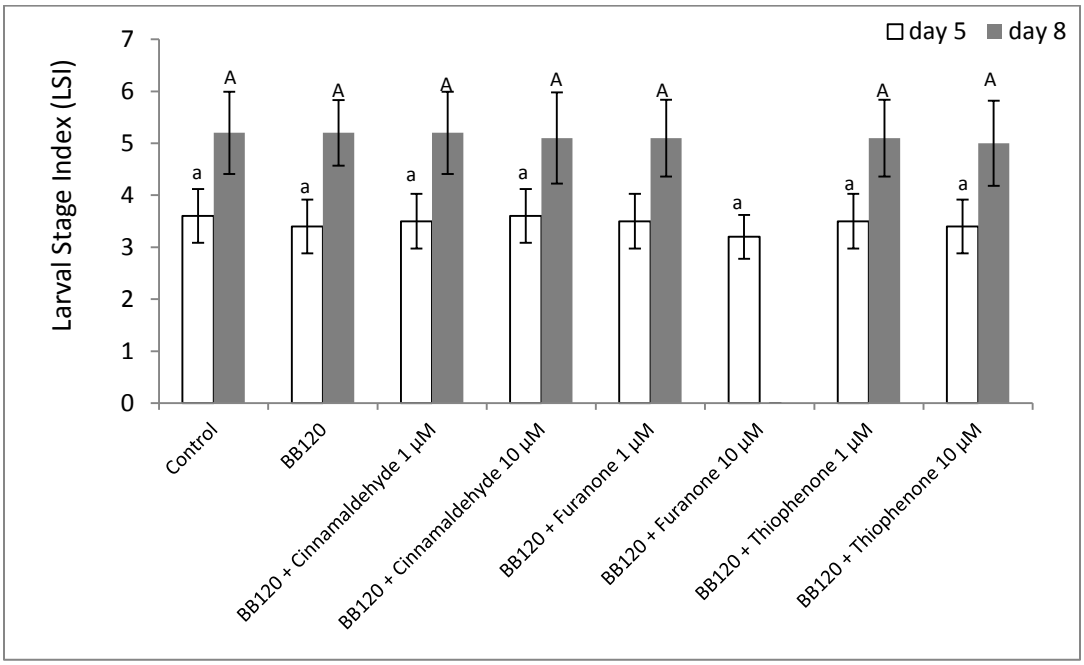


Figure 18. Larval stage Index (LSI) of giant freshwater prawn larvae after 5 and 8 days of challenge with *Vibrio campbellii* BB120, with and without quorum sensing-disrupting compounds (average ± standard deviation of five replicates). Bars with different letter are significantly different from each other (p<0.05). Cinnamaldehyde, the furanone (*Z*-)-4-bromo-5-(bromomethylene)-2(5*H*)-furanone, and the thiophenone (*Z*)-4-((5-(bromomethylene)-2-oxo-2,5-dihydrothiophen-3-yl)methoxy)-4-oxobutanoic acid were added to the culture water at the start of the experiment at 1 and 10 µM.

Brominated furanones have been reported before to protect brine shrimp larvae from pathogenic *V. campbellii* (Defoirdt et al., 2006a) and rainbow trout (*Oncorhyncus mykiss*) from *Vibrio anguillarum* (Rasch et al., 2004), and the thiophenone compound has been reported before to protect brine shrimp larvae from *V. campbellii* (Defoirdt et al., 2012). As

far as we know, this study provides the first evidence of a protective effect of this kind of compounds in a commercial crustacean species. The concentrations at which the compounds protected the giant freshwater prawn larvae were lower than those needed to protect brine shrimp for all three compounds tested (Defoirdt et al., 2006a; Brackman et al., 2008; Defoirdt et al., 2012). This might indicate that the vibrios are more vulnerable to quorum sensing disruption during infection of giant freshwater prawn larvae when compared to brine shrimp. However, we cannot exclude the possibility that in addition to quorum sensing disruption, the compounds have other (yet unknown) activities towards either the larvae or the bacteria.

Our results are consistent with previous reports showing that cinnamaldehyde is able to protect brine shrimp larvae against *V. campbellii* (Brackman et al., 2008) and burbot (*Lota lota L*.) larvae from *Aeromonas hydrophila* and *Aeromonas salmonicida* (Natrah et al., 2012). The concentrations of cinnamaldehyde used in this study have no effect on larval growth and also consistent as described before by Brackman et al. (2008). The observation that higher levels of furanone resulted in high mortality of the prawn larvae is also consistent with the literature, in that this kind of compounds is known to have considerable toxicity towards higher organisms (Hentzer & Givskov, 2003; Defoirdt et al., 2006a). Apparently, giant freshwater prawn larvae are less tolerant to the furanone than brine shrimp larvae, which still survived at ~80 µM (Defoirdt et al., 2006a). As brominated furanones and brominated thiophenones are highly reactive molecules, we previously hypothesised that inactivation of essential proteins (caused by binding of the compounds to nucleophilic amino acid residues) is responsible for their toxicity, and that the presence of a side chain in the thiophenone compound reduces toxicity by limiting the access to some nucleophilic acid residues of essential proteins (due to steric hindrance) (Defoirdt et al., 2012). Indeed, the presence of a 3-alkyl side chain has been reported to result in lower toxicity of brominated furanones to planktonic bacterial cells, without major impact on quorum sensing-disrupting activity (Steenackers et al., 2010).

In conclusion, in this study, we found that the use of quorum sensing-disrupting compounds is a valid strategy to protect giant freshwater prawn larvae from *V. campbellii* without negative effect on growth of the larvae. Further developing this kind of treatments will hopefully lead to a sustainable production of aquatic larvae in general and those of the giant freshwater prawn in specific.

ACKNOWLEDGEMENTS

We thank Bonnie Bassler for providing us with *V. campbellii* BB120. We also thank Gunnar Herstad for performing the synthesis of the brominated thiophenone. This work was funded by the Research Foundation of Flanders (FWO-Vlaanderen; project no 1.5.013.12N) and by the Directorate General of Higher Education of Indonesia through doctoral scholarship to Pande Gde Sasmita Julyantoro. Tom Defoirdt is a postdoctoral fellow of FWO-Vlaanderen

CHAPTER 5

ISOLATION OF ACYLHOMOSERINE LACTONE-DEGRADING BACTERIA FROM MICRO-ALGAL CULTURES AND THEIR IMPACT ON MICRO-ALGAL GROWTH AND ON THE VIRULENCE OF PATHOGENIC *VIBRIO CAMPBELLII* TO THE LARVAE OF GIANT FRESHWATER PRAWN *MACROBRACHIUM ROSENBERGII*

Redrafted after:
Pande GSJ, Natrah FMI, Flandez AVB, Kumar U, Niu Y, Bossier P, Defoirdt T (2015) Isolation of AHL-degrading bacteria from micro-algal cultures and their impact on algal growth and on virulence of *Vibrio campbellii* to prawn larvae. Applied Microbiology and Biotechnology, in press.

ABSTRACT

Inactivation of the quorum sensing (QS) signal molecules, such as acylhomoserine lactones (AHLs) of pathogenic bacteria has been proposed as a novel method to combat bacterial diseases in aquaculture. AHL-degrading bacteria have been isolated from various aquatic environments. However, despite the importance of micro-algae for aquaculture, AHL degradation by bacteria associated with micro-algal cultures has thus far not been investigated. In this study, we isolated AHL-degrading bacteria from open cultures of the microalgae *Tetraselmis suecica* and *Chaetoceros muelleri*, and tested their influence on algal growth and their ability to protect prawn larvae from *V. campbellii*. We isolated *Pseudomonas* sp. NFMI-T and *Bacillus* sp. and NFMI-C from *Tetraselmis suecica* and *Chaetocheros muelleri*, respectively. An AHL degradation assay showed that either monocultures or co-cultures of the isolates were able to degrade the AHL N-hexanoyl-L-homoserine lactone. In contrast, *Bacillus* sp. NFMI-C, but not *Pseudomonas* sp. NFMI-T, was able to interfere with quorum sensing-regulated bioluminescence in *V. campbellii*. Both isolates were able to persist for up to 3 weeks in conventionalised microalgal cultures, indicating that they were able to establish and maintain themselves within open algal cultures. Using gnotobiotic algal cultures, we found that the isolates did not affect growth of the micro-algae from which they were isolated, whereas a mixture of both isolates increased the growth of *Tetraselmis*, whereas it decreased the growth of *Chaetoceros.* Finally, addition of *Bacillus* sp. NFMI-C to the rearing water of giant freshwater prawn (*Macrobrachium rosenbergii*) larvae significantly improved survival of the larvae when challenged with pathogenic *V. campbellii,* whereas it had no effect on larval growth.

Keywords: quorum quenching, acylase, lactonase, *Macrobrachium rosenbergii*, vibriosis

INTRODUCTION

Aquaculture, the farming of aquatic animals and plants (algae) in marine, brackish and freshwater environments, is the fastest-growing food-producing industry worldwide (Bostock et al., 2010). The giant freshwater prawn, *Macrobrachium rosenbergii* (de Man, 1879), is an important crustacean species from an economic perspective, with an annual global production of more than 200 000 tons (New et al., 2010; FAO, 2013). Disease outbreaks are considered to be amongst the major obstacles to produce healthy and high quality seed for the further expansion of giant freshwater prawn culture (Nhan et al., 2010). Previous studies have shown that *Vibrio spp*., including *Vibrio campbellii*, are a major cause of diseases in the early life stages (larvae and postlarvae), all of which need brackish water to survive (Tonguthai, 1997; Kennedy et al., 2006; New et al., 2010; FAO, 2013).

Bacterial diseases in aquaculture have thus far mainly been tackled by using antibiotics, but unfortunately, the use of these compounds has not been very successful and has led to the development and spread of resistant pathogens (Defoirdt et al., 2011a). Therefore, there is a need for novel methods to control bacterial disease. As virulence gene expression in many bacterial pathogens has been reported to be controlled by quorum sensing, interference with this cell-to-cell communication mechanism has been proposed as a novel biocontrol strategy (Defoirdt et al., 2004). *Vibrio campbellii* contains a three-channel quorum sensing system, with three different types of signal molecules (HAI-1, AI-2 and CAI-1, respectively) feeding a common signal transduction cascade (Ruwandeepika et al., 2012). Quorum sensing has been reported to control the expression of different virulence genes in *Vibrio campbellii* and we recently reported that the HAI-1 and the AI-2 mediated channels of the *Vibrio campbellii* quorum sensing system are essential for full virulence to giant freshwater prawn larvae (Pande et al., 2013a).

The use of signal molecule-degrading bacteria is one of the most intensively studied strategies to interfere with quorum sensing. The ability to inactivate acylhomoserine

lactones (AHLs), one of the types of quorum sensing molecules, is widely distributed in the bacterial kingdom (Dong et al., 2007). Two major classes of AHL-inactivating enzymes have been described: lactonases, which are e.g. produced by *Bacillus* spp., cleave the lactone ring of the signal molecules, whereas acylases, e.g. produced by *Pseudomonas* spp., cleaves the AHL molecule into homoserine lactone and a fatty acid (Fast & Titpon, 2012). As *Vibrio campbellii* HAI-1 is an AHL and as this signal is essential for full virulence towards giant freshwater prawn larvae, the use of AHL-degrading bacteria might be an effective strategy to protect the larvae from the pathogen. This kind of bacteria has been isolated from various environments, including the digestive tract of healthy shrimp (Tinh et al., 2007) and fish (Cam et al., 2009a).

Micro-algae are an important constituent of many aquaculture systems, especially the so-called greenwater systems, in which the animals are cultured in water containing high levels of micro-algae (Hargreaves, 2006). According to FAO, different density of microalgae have been used in this green water system for rearing larvae of many aquaculture animals, such as gilthead seabream *Sparus aurata* (50000 cells ml^{-1} of *Isochrysis* sp. and 400000 $cells.ml^{-1}$ of *Chlorella* sp. per day), milkfish *Chanos chanos* (between 500 and 3500 cells.ml-1 *Chlorella* are added from hatching until day 21), Mahimahi *Coryphaena hippurus* (200000 cells.ml-1 of either *Chaetoceros gracilis*, *Tetraselmis chui*, or *Chlorella* sp.), halibut *Hippoglossus hippoglossus* (*Tetraselmis* sp.), and turbot *Scophthalmus maximus* (60000 $cells.ml^{-1}$ of *Tetraselmis* sp. or 130000 $cells.ml^{-1}$ of *I. galbana*) (Lavens & Sorgeloos, 1996).

Indeed, greenwater systems are used to culture various aquaculture animals, including giant freshwater prawn larvae (FAO, 2013). Micro-algal cultures and greenwater used in aquaculture are not axenic and contain high levels of bacteria. However, the potential beneficial effects of bacteria associated with micro-algae remain largely unexplored (Natrah et al., 2013). Although much more work is needed to fully understand complex interactions between microalgae and bacteria, the exploration of these interactions are very important

to develop and improve some biotechnological processes (Kouzuma & Watanabe, 2015). In this study, we aimed at investigating (1) whether AHL-degrading bacteria can be isolated from microalgal cultures, (2) whether they have any impact on algal growth, and (3) whether they can protect giant freshwater prawn larvae against *V. campbellii*.

MATERIALS AND METHODS

Microalgal strains and culture conditions

Axenic *Tetraselmis suecica* CCAP66/4 and *Chaetoceros muelleri* CCMP1316 were obtained from the Culture Collection of Algae and Protozoa (CCAP, Dunstaffnage Marine Laboratory, Scotland) and the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP, USA), respectively. The algae were grown in Guillard's F/2 medium (Sigma) (with silicate addition for *Chaetoceros muelleri*) in sterile 250 ml Schott bottles provided with 0.22 µm filtered aeration. All parameters for algal culture were kept constant (pH 7, continuous light of 100 µmol photons.$m^{-2}sec^{-1}$, temperature of 24^{o}C and 30 g.l^{-1} salinity). The density of axenic cultures was measured using a Bürker hemocytometer and a spectrophotometer (Thermo Spectronic) at OD_{550} nm. Axenity tests were done by plating the supernatant on marine agar and Luria Bertani (LB) agar 30 g.l^{-1} salinity (bacterial contamination test) and potato dextrose agar (fungal contamination test). Samples were also routinely checked microscopically at 1000x magnification with oil immersion, immediately before harvesting.

Isolation of AHL-degrading bacteria from microalgal cultures

Open cultures of *Tetraselmis suecica* and *Chaetoceros muelleri* were grown under similar conditions as described above for strains CCAP66/4 and CCMP1316. Fifty microlitres of the cultures were transferred to sterile erlenmeyer flasks containing 5 ml of sterile NaCl solution (30 g.l^{-1}) containing 50 mg.l^{-1} *N*-hexanoyl-L-homoserine lactone (HHL). The erlenmeyer flasks were covered with aluminium foil to prevent the growth of the micro-algae and were incubated on a shaker (120 rpm) at 24°C. The isolation was performed in

four consecutive cycles (72h for the first cycle and 48h for the second to fourth cycle). At the end of each cycle, 50 µl of the suspension was transferred to a new flask. At the end of the fourth cycle, the suspensions were spread-plated on Luria-Bertani agar containing 30 $g.l^{-1}$ NaCl (LB_{30}) and after 48h incubation at 24°C, colonies were picked, suspended in a 30 $g.l^{-1}$ NaCl solution and plated again. After 3 rounds of purification, isolates were grown in LB_{30} broth for 24h at 24°C and grown cultures were stored at -80°C in 40% glycerol. Two isolates, NFMI-T (GenBank accession number KM525667) and NFMI-C (GenBank accession number KM525666) isolated from *Tetraselmis suecica* and *Chaetoceros muelleri*, respectively, were used in further experiments.

Bacterial strains and culture conditions

Vibrio campbellii BB120 (Bassler et al., 1997), its mutant JAF548 (Freeman & Bassler, 1999) and the AHL-degrading isolates were stored at -80^{o}C in 40% glycerol. The stocks were streaked onto LB agar and after 24 hours of incubation at 28^{o}C, a single colony was picked and inoculated into 5 ml fresh LB broth and incubated overnight at 28^{o}C under constant agitation (100 min^{-1}). For the preparation of inocula for giant freshwater prawn challenge tests, strains were grown in LB broth containing 12 $g.l^{-1}$ NaCl (LB_{12}).

Selection of rifampicin resistant mutants of the isolates

Rifampicin resistant mutants of the isolates were selected as described in Pande et al. (2013a). Briefly, 100 µl of densely grown cultures (OD_{600} of 1) were inoculated into 5 ml of fresh LB broth containing 50 $mg.l^{-1}$ rifampicin (Sigma) and incubated for 5 days at 24^{o}C under constant agitation (100 min^{-1}). The grown cultures were inoculated into fresh LB broth with 50 $mg.l^{-1}$ rifampicin. The grown cultures were stored at -80^{o}C in 40% glycerol until use.

Quantification of *N*-hexanoyl-L-homoserine lactone (HHL)

A stock solution of HHL was prepared by dissolving HHL (Fluka) in 200 µl of ethanol (95%) and then further diluted to a final concentration of 2500 $mg.l^{-1}$ by adding sterile distilled water. A plate diffusion method was used for quantitative detection of HHL using *Chromobacterium violaceum* CV026 as a reporter (Defoirdt et al., 2011b). Briefly, CV026 was grown to an optical density of around 2 at 550 nm in buffered (pH 6.5) LB medium containing 20 $mg.l^{-1}$ kanamycin and spread over buffered (pH 6.5) LB plates. Subsequently, 10 µl of sample solution was applied to the centre of the plates and the plates were incubated at 28°C for 48h. After the incubation, the zone of purple-pigmented CV026 was measured and the concentration of HHL in the sample was calculated based on a standard curve.

AHL degradation assay

AHL degradation by the isolates was studied as reported previously (Defoirdt et al., 2011b). Briefly, the isolates (either single isolates or a 1:1 mixture) were inoculated at 10^8 $CFU.ml^{-1}$ in buffered LB_{30} medium (pH 6.5) supplemented with 10 $mg.l^{-1}$ HHL. At regular time intervals, 1 ml samples from each culture were taken and filtered over a 0.2 µm filter. The HHL concentration in the cell-free supernatants was determined as described above using *C. violaceum* CV026. *Pseudomonas* sp. P3/pME6000 and *Pseudomonas* sp. P3/pME6863 (= pME6000 + the *Bacillus* AHL lactonase gene *aiiA*; Molina et al., 2003), grown under the same conditions, were used as negative and positive control, respectively.

Identification of the isolates by 16S rRNA gene sequencing

PCR targeting a 1500 base pair fragment of the 16S rRNA gene of the isolates was performed according to Boon et al. (2002) using the primer pair GM3f and GM4r (Biolegio, Nijmegen, The Netherlands). PCR was performed with a Gene Amps PCR system 2700 thermal cycler (PE Applied Biosystems, Nieuwerkerkena/d Ijssel, The Netherlands) using the

program: 95 °C for 5 minutes, 32 cycles of 94 °C for 1 minute, 42 °C for 1 minutes, 72 °C for 3 minutes and finally an extension period of 72°C for 10 minutes. DNA sequencing of the obtained PCR products was carried out at IIT Biotech (Bielefield, Germany). The nucleotide sequences of the isolates were deposited in the Genbank database (http://www.ncbi.nlm.nih.gov/Genbank). Homology searches were completed with the BLAST server of the National Centre for Biotechnology Information for the comparison of the nucleotide query sequence against a nucleotide sequence database (blastn).

Impact of the isolates on the growth of gnotobiotic micro-algae

Axenic *Tetraselmis suecica* CCAP66/4 and *Chaetoceros muelleri* CCMP1316 were inoculated in 250 ml Erlenmeyer flasks containing 50 ml F/2 medium (with silica for *Chaetoceros muelleri*) at 10^4cells per ml, with and without the isolates (either single isolates or a 1:1 mixture) at 10^2 CFU.ml^{-1}. As controls, the isolates were inoculated in flasks without algae. The flasks were incubated on a shaker (120 rpm) with constant illumination 100 µmol photons.m^{-2}sec^{-1} at 24°C for 15 days. All treatments were performed in triplicate. Growth of the micro-algae was monitored by measuring *in vivo* chlorophyll afluorescence (excitation 430 nm, emission 670 nm) using a Tecan Infinite 200 microplate reader (Tecan, Mechelen, Belgium).

Persistence of the isolates in conventionalised microalgal cultures

Conventionalised cultures of *Tetraselmis suecica* CCAP66/4 and *Chaetoceros muelleri* CCMP1316 were obtained by inoculating axenic cultures with 10^5 CFU.ml^{-1} of microbiota taken from the open cultures of the respective microalgae (obtained by taking the supernatant of cultures centrifuged at 300 x *g* for 5 minutes). The micro-algae were inoculated in 250 ml Erlenmeyer flasks containing 50 ml F/2 medium (with silica for *Chaetoceros muelleri*) at 10^4cells per ml. A 1:1 mixture of the rifampicin resistant mutants of NFMI-C and NFMI-T was inoculated at the start of the experiment at a total density of 10^5 CFU.ml^{-1}. The flasks were incubated on a shaker (120 rpm) with constant illumination

100 µmol photons.$m^{-2}sec^{-1}$ at 24°C. All treatments were performed in triplicate. The density of the AHL degraders was determined after 2 and 3 weeks of incubation by plating on LB_{30} agar containing 100 $mg.l^{-1}$ rifampicin. Conventionalised algal cultures without the addition of the isolates were used as controls.

Giant freshwater prawn challenge test

Giant freshwater prawn challenge tests were performed as described in **Chapter 3**. Larvae were challenged by adding 10^6 CFU.ml^{-1} of *V. campbellii* BB120 to the rearing water on the day after first feeding, and *Bacillus* sp. NFMI-C was added at 10^5 CFU.ml^{-1}. Survival was counted daily in the treatment receiving *V. campbellii* BB120 only, and the challenge test was stopped when more than 50% mortality was achieved. At this time point, larval survival was determined in all treatments by considering that only those larvae presenting movement of appendages were alive.

Statistical data analysis

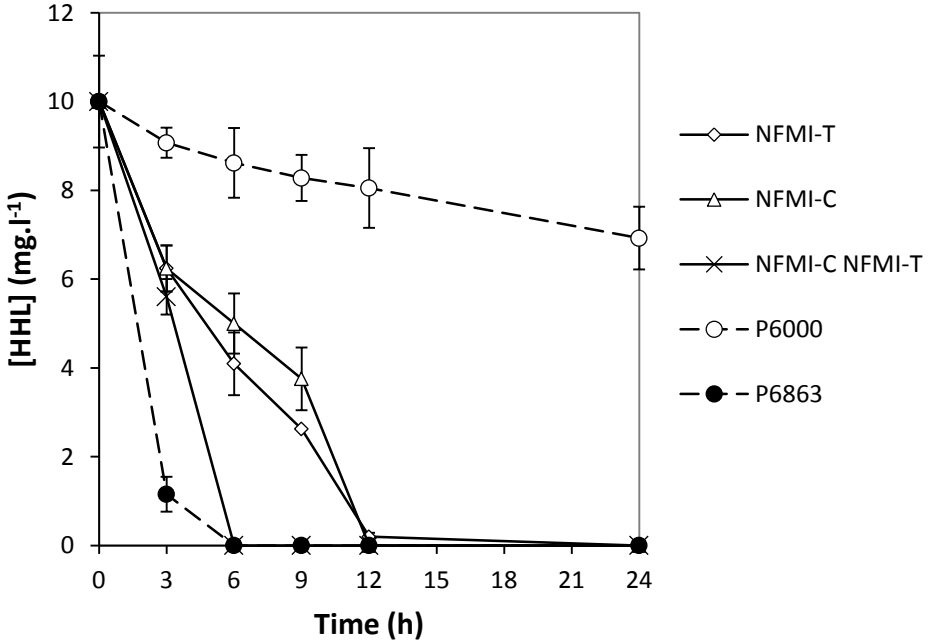
Statistical analyses were performed using the SPSS software, version 20. Giant freshwater prawn survival data were arcsin transformed in order to satisfy normal distribution and homoscedasticity requirements. Data were analyzed by one way ANOVA, followed by Tukey multiple range tests with a significance level set at 0.05.

RESULTS

Isolation of AHL-degrading bacteria from micro-algal cultures

AHL-degrading strains were isolated from open cultures of *Tetraselmis suecica* and *Chaetoceros muelleri* by sequentially culturing in a medium containing *N*-hexanoyl-L-homoserine lactone (HHL) as the sole carbon and nitrogen source. Two isolates, NFMI-T and NFMI-C (isolated from *Tetraselmis suecica* and *Chaetoceros muelleri*, respectively), were used in further experiments. The isolates were inoculated at 10^8 CFU.ml^{-1} in buffered LB

medium supplemented with 10 mg.l^{-1} HHL in order to determine whether they were able to degrade AHLs in a nutrient-rich background. Both isolates were able to degrade HHL to below detection limit within 12h when grown in monoculture (**Figure 19**). However, degradation proceeded faster when inoculated in a 1:1 mixture, with the HHL level being decreased to below detection limit (0.5 mg.l^{-1}; Cam et al., 2009a) after 6h. The HHL degradation rates of NFMI-C monoculture, NFMI-T monoculture and an NFMI-C NFMI-T coculture were 0.75, 0.77 and 1.67 mg.l^{-1}.h^{-1}, respectively. The first isolation and AHL degradation assay was performed by Natrah FMI at laboratorium of Aquaculture and *Artemia* Reference Center, Ghent University.



.

Figure 19. *N*-hexanoyl-L-homoserine lactone (HHL) degradation by the isolates NFMI-C and NFMI-T, either alone or as a 1:1 mixture, in Luria-Bertani broth containing 10 mg.l^{-1} HHL. *Pseudomonas* sp. P3/pME6000 was used as a negative control; *Pseudomonas* sp. P3/pME6863 (containing the *Bacillus* sp. AHL lactonase gene *aiiA*) was used as a positive control. All strains were inoculated at a total density of 10^8 CFU.ml^{-1}. This experiment was performed by F.M.I. Natrah.

Identification of the isolates

The isolates were identified by sequencing of the 16S rDNA. An NCBI BLAST search revealed that isolate NFMI-C was most closely related to *Bacillus* spp. and that isolate NFMI-T was most closely related to *Pseudomonas* spp. (**Table 6**).

Table 6. Identification of the isolates. Closest relatives based on NCBI BLAST performed using partial 16S rDNA sequences. The identification was done by T. Defoirdt.

Isolate	Source	Accession n°[1]	Closest relative (accession n°)	Similarity (%)
Bacillus sp. NFMI-C	*Chaetoceros muelleri*	KM525666	*Bacillus cereus* LNE7 (AM397642.1)	100
Pseudomonas sp. NFMI-T	*Tetraselmis suecica*	KM525667	*Pseudomonas putida BACD4* (KF413409.1)	99

[1] GenBank accession numbers

Impact of the isolates on growth of gnotobiotic micro-algae

Axenic *Tetraselmis suecica* CCAP66/4 and *Chaetoceros muelleri* CCMP1316 were inoculated in fresh medium, with and without the isolates (either single isolates or a 1:1 mixture) and growth of the micro-algae was monitored by measuring *in vivo* chlorophyll a fluorescence. *Pseudomonas* sp. NFMI-T alone had no effect on the growth of *Tetraselmis suecica* CCAP66/4, whereas addition of a mixture of both isolates resulted in higher chlorophyll fluorescence after 12 and 15 days of culture (**Figure 20A**). In case of *Chaetoceros muelleri* CCMP1316, growth was not affected by *Bacillus* sp. NFMI-C alone, whereas the mixture of both isolates resulted in decreased chlorophyll fluorescence throughout the experiment (**Figure 20B**). Because differences could only be observed when using both isolates and because the mixture showed the best HHL degrading capacity, a 1:1 mixture of the isolates was used in further coculture experiments with micro-algae.

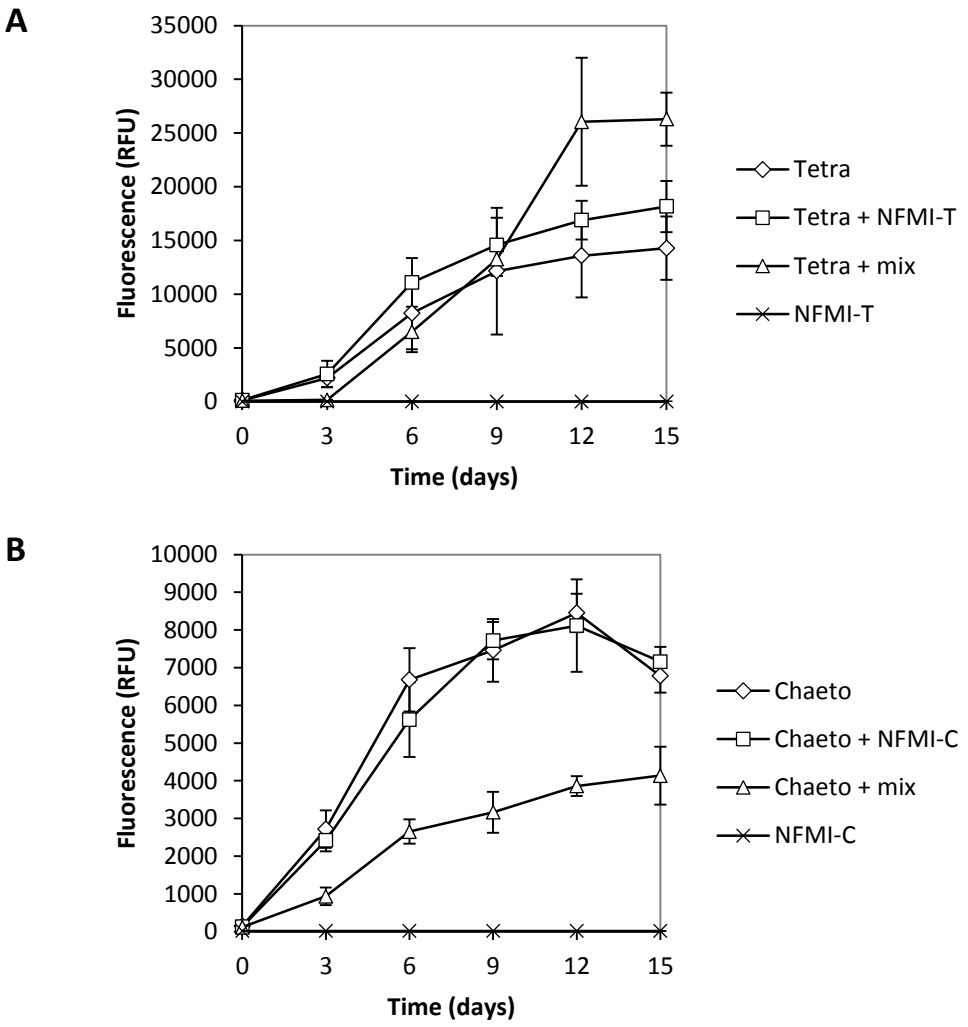


Figure 20. Impact of the isolates on growth of the micro-algae in Guillard's F/2 medium. (**A**) Chlorophyl fluorescence of *Tetraselmis suecica* CCAP66/4*,* with or without *Pseudomonas* sp. NFMI-T or a 1:1 mixture of both isolates. (**B**) Chlorophyl fluorescence of *Isochrysis* sp. CCAP927/14*,* with or without *Bacillus* sp. NFMI-I or a 1:1mixture of both isolates. Cultures of the isolates without micro-algae were used as controls. This experiment was performed by F.M.I. Natrah.

Persistence of the isolate mixture in conventionalized microalgal cultures

Conventionalised cultures of *Tetraselmis suecica* CCAP66/4 and *Chaetoceros muelleri* CCMP1316 were obtained by inoculating axenic cultures with 10^5 CFU.ml^{-1} of microbiota taken from the open cultures of the respective microalgae. A 1:1 mixture of natural rifampicin resistant mutants of *Bacillus* sp. NFMI-C and *Pseudomonas* sp. NFMI-T was inoculated at the start of the experiment at a total density of 10^5 CFU.ml^{-1} and the density of the AHL degraders was determined after 2 and 3 weeks of incubation by plating on LB_{30} agar containing 100 mg.l^{-1} rifampicin. The isolates were detected after 2 and 3 weeks of culture in both conventionalized *Tetraselmis suecica* CCAP66/4 and *Chaetoceros muelleri* CCMP1316 cultures (**Table 7**), where they had increased to approximately 10^6 CFU.ml^{-1}.

Table 7. Persistence of the 1:1 mixture of the AHL-degrading isolates NFMI-C and NFMI-T in conventionalized microalgal cultures after 2 and 3 weeks of culture. Plate counts on LB agar containing 100 mg.l^{-1} rifampicin. The conventionalized microalgal cultures were obtained by inoculating axenic cultures with 10^5 CFU.ml^{-1} of microbiota taken from open cultures of the respective microalgae. A 1:1 mixture of rifampicin resistant isolates was inoculated at the start of the experiment at a total density of 10^5 CFU.ml^{-1}.

Treatment	**Plate counts (x 10^6 CFU.ml^{-1})**	
	Week 2	**Week 3**
Conventionalised *Tetraselmis suecica* CCAP66/4+ isolates	1.1 ± 0.2	1.2 ± 0.9
Conventionalised *Tetraselmis suecica* CCAP66/4	ND	ND
Conventionalised *Chaetoceros muelleri* CCMP1316 + isolates	1.7 ± 0.2	1.6 ± 0.1
Conventionalised *Chaetoceros muelleri* CCMP1316	ND	ND

ND: Not Detected

Impact of the isolates on AHL quorum sensing in *Vibrio campbellii*

Because bioluminescence is one of the phenotypes that are regulated by quorum sensing in *V. campbellii*, we used bioluminescence as a read-out of quorum sensing activity and determined the impact of the isolates on bioluminescence of wild type *V. campbellii* BB120. In order to compensate for the competition for nutrients, we mixed BB120 with its mutant JAF548 as a control. JAF548 has a completely inactive quorum sensing system (and therefore is not luminescent; Freeman & Bassler, 1999). The results revealed that *Bacillus* sp. NFMI-C, but not *Pseudomonas* sp. NFMI-T, decreased quorum sensing-regulated luminescence of *V. campbellii* in co-culture (**Figure 21**).

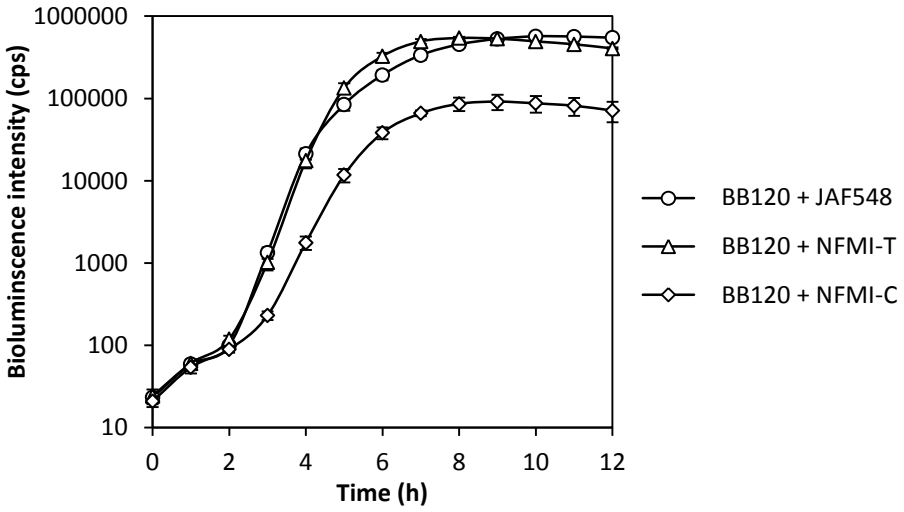


Figure 21. Quorum sensing-regulated bioluminescence of *V. campbellii* BB120 in co-culture with the AHL degrading isolates NFMI-C or NFMI-T. A co-culture with the dark mutant of BB120, JAF548, served as control. Error bars represent the standard deviation of 6 replicates.

However, there were no such effects when *V. campbellii* was grown in the presence of cell-free supernatants of the isolate (data not shown). Importantly, neither of the isolates affected the growth of *V. campbellii* in co-culture (**Table 8**), nor did cell-free supernatants of the isolates affect growth of the pathogen.

Table 8. Impact of the isolates on cell density of *V. campbellii* BB120 after 12 hours of coculture as determined by plate counting of luminescent cells.

Treatment	Cell density (x 10^8 CFU.mL^{-1})
BB120 + JAF548	4.7 ± 0.5
BB120 + NFMI-C	4.6 ± 0.2
BB120 + NFMI-T	4.5 ± 0.2

Impact of *Bacillus* sp. NFMI-C on the survival and growth of giant freshwater prawn larvae challenged with *Vibrio campbellii*

Our previous research showed that AHL quorum sensing is essential for full virulence of *V. campbellii* towards giant freshwater prawn larvae (Pande et al., 2013a). Hence, since *Bacillus* sp. NFMI-C was able to interfere with quorum sensing in *V. campbellii*, we went further to investigate whether this isolate was able to protect giant freshwater prawn larvae from the pathogen. Addition of *Bacillus* sp. NFMI-C to the rearing water indeed resulted in a significantly improved survival of challenged prawn larvae (**Table 9**). Consistent with our previous work, we found no difference in growth (as assessed by determining the larval stage index) between the different treatments.

Table 9. Survival and growth (as expressed by the larval stage index-LSI) of giant freshwater prawn larvae after 6 days of challenge with *V. campbellii* BB120 (average ± standard deviation of 5 prawn cultures). "Control" refers to unchallenged larvae that were otherwise treated in the same way as the other larvae.

Treatment	Survival (%)	LSI
Control	83 ± 7^c	4.4 ± 0.5^a
BB120	42 ± 8^a	4.4 ± 0.5^a
BB120 + NFMI-C	67 ± 5^b	4.6 ± 0.5^a

DISCUSSION

The ability to interfere with bacterial quorum sensing by degrading AHL molecules is widely distributed in the bacterial kingdom (LaSarre & Federle, 2013), and because of their potential as novel disease control agents, AHL-degrading bacteria have been isolated from various aquatic environments, including the water column, sediment, seaweed and the intestinal tract of healthy aquatic organisms (Tang & Zhang, 2014). However, despite the importance of micro-algae for e.g. aquaculture (Natrah et al., 2013), AHL degradation by bacteria associated with micro-algal cultures has thus far not been investigated. In this study, we report the isolation of two AHL-degrading strains, *Bacillus* sp. NFMI-C and *Pseudomonas* sp. NFMI-T, from open cultures of *Tetraselmis suecica* and *Chaetoceros muelleri,* respectively. Both strains showed HHL degradation rates that were similar to those of *Bacillus* sp. strains isolated from the intestinal tract of shrimp and fish (0.7-0.9 $mg.l^{-1}.h^{-1}$) (Defoirdt et al., 2011b). However, a co-culture of both strains degraded HHL approximately twice as fast. HHL was used as test compound because it is relevant to aquaculture. Indeed, it is produced by pathogenic bacteria such as *Aeromonas hydrophila, Aeromonas salmonicida, Edwardsiella tarda* and *Vibrio salmonicida* (Swift et al., 1997; Morohoshi et al., 2004; Bruhn et al., 2005). Furthermore, *Bacillus* sp. NFMI-C was also found to interfere with quorum sensing-regulated bioluminescence in *V. campbellii* (supposedly by degradation of the AHL *N*-3-hydroxybutanoyl-L-homoserine lactone) in co-culture.

Several *Bacillus* species have been reported to produce AHL lactonases, which inactivate AHLs by hydrolysing the lactone ring. Lactonases are intracellular enzymes capable of inactivating a wide range of AHLs, varying in acyl chain length and substitution (Dong et al., 2007). *Pseudomonas* species, in contrast, have been reported to produce AHL acylases, which cleave AHLs by aminohydrolysis. Unlike lactonases, acylases exhibit substrate specificity depending on the acyl side chain length and the substitution on the β-position of the acyl chain (Tang & Zhang, 2014). This might explain why *Pseudomonas* sp. NFMI-T was

not able to interfere with quorum sensing in *V. campbellii* although it was able to degrade HHL.

In addition to the AHL degradation capacity of the isolates, we also investigated their impact on micro-algal growth. *Bacillus* sp. NFMI-C and *Pseudomonas* sp. NFMI-T had no effect on growth of *Chaetoceros muelleri* and *Tetraselmis suecica*, respectively, when added alone. However, a mixture of both strains increased the growth of *Tetraselmis suecica,* whereas it decreased the growth of *Chaetoceros muelleri*. Interactions between bacteria and micro-algae are fairly complex and not yet completely understood. The exact mechanisms by which bacteria stimulate micro-algae are largely unknown, although a few compounds responsible for such effects (including vitamins and hormones) have been identified (Natrah et al., 2013). The negative impact of the NFMI-C NFMI-T mixture on the growth of *Chaetoceros muelleri* might be due to competition for nutrients. Indeed, bacteria have been reported to decrease the growth of another diatom, *Cylindrotheca fusiformis*, at low phosphate concentrations (suggesting that the bacteria scavenge phosphate better than the micro-algae) (Amin et al., 2012). Another possibility is that the *Pseudomonas* sp. NFMI-T produces a compound that inhibits the growth of *Chaetoceros,* without affecting the growth of *Tetraselmis* (from which it was isolated). Some bacteria species including *Pseudomonas aeruginosa* has been known to produce an algicidal compound (Natrah et al., 2013). This algicidal activity can be caused either by the release of algicidal compounds in the environment or by lysis of the microalgal cells following attachment (Mayali & Azam 2004).

Our *in vivo* experiment revealed that *Bacillus* sp. NFMI-C significantly increased the survival of giant freshwater prawn larvae challenged with *V. campbellii*, whereas the isolate had no effect on larval growth. It has been demonstrated that the degradation of AHL-mediated quorum sensing could reduce the virulence of aquaculture pathogenic bacteria. Our result is consistent with previous reports showing that AHL degraders are able to improve the survival of prawn larvae (Cam et al., 2009b) and turbot larvae (*Scophthalmus maximus* L.)

(Tinh et al., 2008) in the presence of exogenous AHL (which caused mortality in both species; probably by triggering pathogenicity mechanisms in pathogenic bacteria that were naturally present in the cultures). We found that isolate NFMI-C was most closely related to *Bacillus* spp. Strains belonging to *Bacillus* species such as *B. subtilis*, *B. cereus*, *B. coagulans*, *B. clausii*, *B. megaterium* and *B. licheniformis* are the most frequently used probiotics in aquaculture (Wang et al., 2008). Hence, the use of *Bacillus* sp. strains able to degrade AHL molecules might be an interesting new type of probiotics for aquaculture with a defined mode of action which still need further research (it does not necessarily mean that AHL degradation is the sole beneficial effect). Along this line, AHL-degrading *Bacillus* sp. have been shown to inhibit the protease production, hemolytic activity and biofilm formation of *A. hydrophila* strain YJ-1, and to significantly improve the survival of zebrafish (*Danio rerio*) challenged with this pathogen (Chu et al., 2014).

In conclusion, in this study, we isolated two ALH-degrading strains, *Bacillus* sp. NFMI-C and *Pseudomonas* sp. NFMI-T, from cultures of the microalgae *Chaetoceros muelleri* and *Tetraselmis suecica*, respectively. Both strains were able to quickly degrade AHLs in a nutrient-rich background. The isolates were able to maintain themselves in conventionalised algal cultures, and their impact on algal growth was dependent on the micro-alga that was studied. *Bacillus* sp. NFMI-C, but not *Pseudomonas* sp. NFMI-T, was able to decrease quorum sensing-regulated luminescence of *V. campbellii* and improved the survival of giant freshwater prawn larvae when challenged with *V. campbellii*. These results showed that these AHL-degrading isolates might have potential as novel biocontrol strains for use in aquaculture. Further research will reveal the efficacy of the isolates in different aquaculture host–pathogen systems. Indeed, although *Pseudomonas* sp. NFMI-T was not able to interfere with quorum sensing in *V. campbellii,* it might be well capable of protecting aquatic hosts from bacteria that use another type of AHL molecule such as *Aeromonas* spp. and *Edwardsiella* spp. which use *N*-hexanoyl-L-homoserine lactone (HHL). Furthermore, a

combination of both isolates might even be more effective since a co-culture showed a higher HHL degradation rate than the single cultures of the isolates.

ACKNOWLEDGEMENTS

This work was funded by the Research Foundation of Flanders (FWO-Vlaanderen; project n^{o} 1.5.013.12N), by the Bijzonder Onderzoeksfonds (BOF)-special research fund for finalizing PhD from Ghent University (Code: 01DI0114) and by the Directorate General of Higher Education of Indonesia through a doctoral scholarship to G.S.J.P, by University Putra Malaysia and Malaysian Ministry of Higher Education (MOHE) through doctoral grant to N.F.M.I., T.D is a postdoctoral fellow of FWO-Vlaanderen.

CHAPTER 6

THE CATECHOLAMINE STRESS HORMONES NOREPINEPHRINE AND DOPAMINE INCREASE THE VIRULENCE OF PATHOGENIC *VIBRIO ANGUILLARUM* AND *VIBRIO CAMPBELLII*

Redrafted after
Pande GSJ, Suong NT, Bossier P, Defoirdt T (2014) The catecholamine stress hormones norepinephrine and dopamine increase the virulence of pathogenic *Vibrio anguillarum* and *Vibrio campbellii*. FEMS Microbiology Ecology 90:761-769

ABSTRACT

Obtaining a better understanding of mechanisms involved in bacterial infections is of paramount importance for the development of novel agents to control disease caused by (antibiotic-resistant) pathogens in aquaculture. In this study, we investigated the impact of catecholamine stress hormones on growth and virulence factor production of pathogenic vibrios (i.e. 2 *Vibrio campbellii* strains and 2 *Vibrio anguillarum* strains). Both norepinephrine and dopamine (at 100 μM) significantly induced growth in media containing serum. The compounds also increased swimming motility of the tested strains, whereas they had no effect on caseinase, chitinase and hemolysin activities. Further, antagonists for eukaryotic catecholamine receptors were able to neutralize some of the effects of the catecholamines. Indeed, the dopaminergic receptor antagonist chlorpromazine neutralized the effect of dopamine, and the α-adrenergic receptor antagonists phentolamine and phenoxybenzamine neutralized the effect of norepinephrine, whereas the β-adrenergic receptor antagonist propranolol had limited to no effect. Finally, pretreatment of pathogenic *V. campbellii* with catecholamines significantly increased its virulence towards giant freshwater prawn larvae. However, the impact of catecholamine receptor antagonists on *in vivo* virulence was less clear-cut when compared to the *in vitro* experiments. In summary, our results show that -similar to enteric pathogens- catecholamines also increase the virulence of vibrios that are pathogenic to aquatic organisms by increasing motility and growth in media containing serum.

Key words: host-microbe interaction; microbial endocrinology; vibriosis

INTRODUCTION

Aquaculture is the fastest growing food producing sector (Bostock et al., 2010) and plays an important role in the economic development worldwide. World aquaculture production increased from 47.3 million tons in 2006 to 63.6 million tons in 2011 and accounted for about 42% in the world fish and shellfish supply (FAO, 2012). In view of the still growing human population, the sector will need to continue growing. However, disease outbreaks are still a significant constraint to this growth and lead to huge losses in the aquaculture industry worldwide (Austin & Zhang, 2006; Ruwandeepika et al., 2011; De Schryver et al., 2014). The frequent use of antibiotics in attempts to control problems caused by pathogenic bacteria has led to the development of antibiotic resistance and as a consequence, new strategies to control bacterial infections in aquaculture animals are urgently needed (Karunasagar et al., 1994; Moriarty, 1998; Defoirdt et al., 2011a).

Pathogenic bacteria produce several virulence factors, i.e. gene products that enable them to enter and damage the host. Inhibiting the production of these virulence factors, a strategy that has been termed antivirulence therapy, is an interesting alternative to antibiotics for controlling bacterial infections (Defoirdt, 2013b). In this respect, it is important to obtain a better understanding of mechanisms involved in bacterial infection as this knowledge can lead to the development of novel control agents.

Host stress has been known for a long time to influence the outcome of host-microbe interactions, and this has been associated with a decreased activity of the host defense system (Verbrugghe et al., 2012). The effects of stress on immunological responses are a consequence of the release of glucocorticoids and biogenic amines, and these mechanisms are conserved in vertebrates and invertebrates (Ottaviani & Franceschi, 1996). During the past decades, evidence has been provided that infectious bacteria have evolved specific detection systems for sensing stress hormones produced by their host, and that detection of these stress hormones results in increased virulence of the pathogens (Lyte, 2004).

Previous studies have shown that catecholamine stress hormones can influence growth, motility, biofilm formation and/or virulence of intestinal pathogens such as *E. coli* and *Salmonella spp.* (Verbrugghe et al., 2012). Further, antagonists of adrenergic and dopaminergic receptors of mammals can also block catecholamine-induced effects in bacteria (Sharaff & Freestone, 2011), and it has been hypothesized that cell-to-cell signaling within the mammalian neuroendocrine system actually represents a late horizontal gene transfer from bacteria (Iyer et al., 2004). Interestingly, catecholamine stress hormones have also been found to increase growth and virulence in human pathogenic *Vibrio parahaemolyticus* (Nakano et al., 2007a). Hence, sensing and responding to catecholamines might also be important with respect to vibriosis in aquaculture organisms since aquatic animals (both invertebrates and vertebrates) also produce these compounds (Ottaviani & Franceschi, 1996).

In this study, we investigated the impact of the catecholamine stress hormones dopamine and norepinephrine, and of mammalian catecholamine receptor antagonists on growth and the expression of virulence-related phenotypes in pathogenic vibrios (including swimming motility, caseinase, chitinase and hemolysin activities), and determined the impact of these compounds on the virulence of *Vibrio campbellii* BB120 towards larvae of the commercially important giant freshwater prawn (*Macrobrachium rosenbergii*).

MATERIALS AND METHODS

Bacterial strains and growth conditions

The strains used in this study are listed in **Table 10**. All strains were stored at -80^{o}C in 40% glycerol and the stocks were streaked onto Luria-Bertani agar containing 12 g.L^{-1} Instant Ocean synthetic sea salt (Aquarium Systems Inc., Sarrebourg, France) (LB_{12}). After 24 h of incubation at 28^{o}C, a single colony was inoculated into 5 ml fresh LB_{12} broth and incubated

overnight at 28^{o}C under constant agitation (100 rotations min^{-1}). Cell densities were measured spectrophotometrically at 600 nm.

Table 10. Bacterial strains used in this study.

Strain	Features	References
V. campbellii BB120 (=ATCC BAA-1116)	Pathogenic towards crustaceans (including giant freshwater prawn larvae)	Bassler et al., 1997; Pande et al., 2013a
V. campbellii LMG21363	Pathogenic towards crustaceans	Soto-Rodriguez et al., 2003
V. anguillarum NB10	Pathogenic towards fish and crustaceans	Croxatto et al., 2007
V. anguillarum HI610	Pathogenic towards fish	Samuelsen & Bergh, 2004

Preparation of stress hormone and inhibitor stocks

Stress hormones and inhibitors used in this studyare listed in **Table 11.** All compounds were obtained from Sigma (Germany) and stock solutions were prepared at 5 mM. For all assays, filter sterilized stress hormone stock solutions were added after autoclaving the agar (and cooling down to 50°C) to reach a final concentration of 100 µM. The inhibitors were tested at 50 µM and 100 µM. When organic solvents were used to prepare stock solutions, control treatments were performed using the same volume of the solvent to verify that the observed effects were not due to the solvent.

Table 11. Catecholamines and inhibitors used in this study

Compound	Specificity of inhibitor	Solvent
Dopamine		Water
Norepinephrine		HCl 0.1 N
Chlorpromazine	Dopaminergic	Water
Phentolamine	α-adrenergic (reversible)	Methanol
Phenoxybenzamine	α-adrenergic (irreversible)	DMSO
Propanolol	β-adrenergic	Ethanol

Growth assays

For the bacterial growth assays, a single colony was grown overnight in LB_{12} broth at 28°C, after which the culture was inoculated at a concentration of 10^2 $CFU.mL^{-1}$ into fresh LB_{12} broth containing 30% (v/v) adult bovine serum (Sigma-Aldrich), with and without 50 µM norepinephrine or dopamine. For the experiment using the eukaryotic catecholamine receptor inhibitors, we used different concentration (50 µM and 100 µM) of norepinephrine or dopamine, and 100 µM of the respective inhibitor (i.e. chlorpromazine for dopamine and phentolamine for norepinephrine). Cultures were grown in 96-well plates at 28°C for 48 h, and the turbidity at 600 nm was monitored every hour using a multireader machine (Infinite M200, TECAN, Austria). Growth curves were obtained from triplicate data.

Motility assay

For assessing the effects of the stress hormones on motility, LB_{12} soft agar plates (containing 0.3% agar) were used. Strains were spotted at the center of the plates (10 µL) and the diameter of the motility zones was measured after 24 h of incubation at 28^{o}C (Yang & Defoirdt, 2014).

Caseinase and hemolysin assays

Both assays were performed according to Natrah et al. (2011b). The caseinase assay plates were prepared by mixing equal volumes of autoclaved double strength LB_{12} agar and a 4% skim milk powder suspension (Oxoid, UK), sterilized separately at 121^{o}C for 5 minutes. Clearing zones surrounding the bacterial colonies and colony diameters were measured after 24 h of incubation at 28°C. Hemolytic assay plates were prepared by supplementing autoclaved LB_{12} agar with 5% defibrinated sheep blood (Oxoid, UK). Colony diameters and clearing zones were measured after two days of incubation at 28°C.

Chitinase assay

Colloidal chitin was prepared based on the protocol of Nagpure & Gupta (2012) with some modifications. 250 mg of chitin from shrimp shells (Sigma, Germany) was added into 2.5 mL of 85% phosphoric acid and kept at 5^{o}C for 24 h. Thereafter, 50 mL of tap water was added and the gelatinous white material thus formed was separated by filtration through filter paper. The retained cake was washed with tap water until the filtrate had a pH of 7.0. Chitinase assay plates were prepared by mixing autoclaved LB_{12} agar with 0.1% (v/v) of the colloidal chitin. After two days of incubation at 28^{o}C, colony diameters and clearing zones were determined and the ratio between both was calculated as a measure for chitinase activity.

Pre-treatment of *V. campbellii* BB120 for giant freshwater prawn challenge tests

In order to avoid any direct effect on the giant freshwater prawn larvae, *V. campbellii* was pretreated with the catecholamines (and inhibitors), after which the compounds were washed away prior to addition to the rearing water. Briefly, *V. campbellii* was grown in LB_{12} broth in the presence of hormones for 16h, and subsequently incubated for 4 h in the presence of norepinephrine (with and without phentolamine) or dopamine (with and without chlorpromazine). After incubation, the cultures were centrifuged at 5000 rpm for 5

minutes. The supernatant was removed and bacteria were re-suspended in autoclaved brackish water (12 $g.l^{-1}$ Instant Ocean), supplemented with 10% of LB_{12}. Untreated *V. campbellii* cultures treated in the same way were used as controls. The bacteria were inoculated into the prawn rearing water at 10^6 $CFU.mL^{-1}$.

Axenic hatching of brine shrimp for prawn larval feeding and giant freshwater prawn larval challenge tests

The materials and methods for axenic hatching and giant freshwater prawn larval challenge test were performed similarly as described in **Chapter 3**.

Statistical analysis

The bacterial swimming motility data were analyzed using independent-samples T-tests, while the data from the *in vitro* experiment of catecholamine inhibitors and *in vivo* challenge test data were analyzed using one-way ANOVA. Significant differences between individual samples were determined using Tukey's post hoc test. Survival data of prawn larvae were arcsin transformed to satisfy normal distribution and homocedasticity requirements. All statistical analyses were done using the SPSS program version 20.

RESULTS

Impact of norepinephrine and dopamine on virulence factor activities of the vibrios *in vitro*

In order to investigate the impact of the catecholamines on swimming motility of pathogenic vibrios, we tested the effect of 100 µM norepinephrine or dopamine on motility of *V. campbellii* BB120, *V. campbellii* LMG 21363, *V. anguillarum* HI610 and *V. anguillarum* NB10 on soft agar. The catecholamines significantly increased swimming motility in all strains tested (**Table 12**).

Table 12. Diameter of the swimming motility halo (mm) of different pathogenic vibrios after 24 h incubation, with and without 100 μM dopamine or norepinephrine (average ± standard deviation of 5 replicates).

Strain	Motility halo diameter (mm)		
	Control	Dopamine	Norepinephrine
V. campbellii BB120	12 ± 1	46 ± 1*	55 ± 1*
V. campbellii LMG21363	26 ± 1	45 ± 3*	69 ± 1*
V. anguillarum HI610	22 ± 1	42 ± 1*	67 ± 1*
V. anguillarum NB10	30 ± 1	51 ± 1*	75 ± 1*

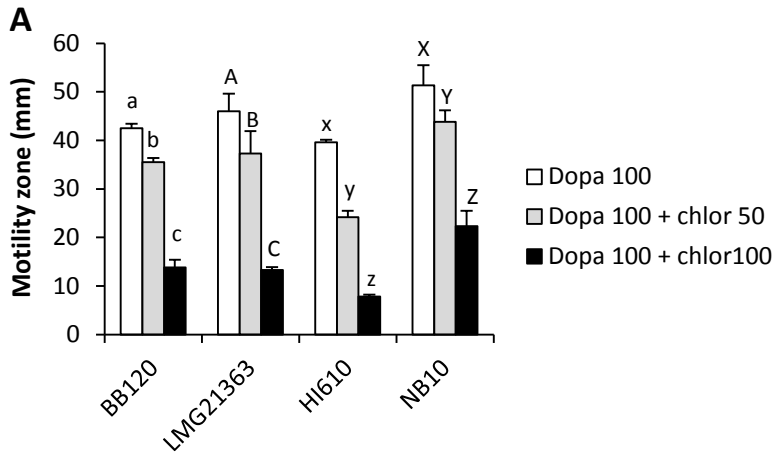
Asterisks indicate significant differences ($p<0.01$) when compared to the respective control

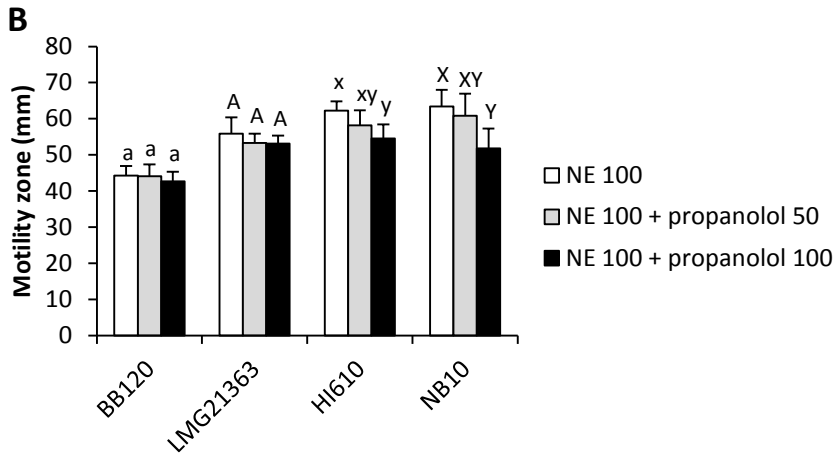
Eukaryotic catecholamine receptor antagonists have been reported to neutralize catecholamine-induced effects in enteric pathogens (Freestone et al., 2007) and therefore, we investigated the impact of eukaryotic catecholamine receptor antagonists on catecholamine-induced motility in the four test strains. The dopaminergic receptor antagonist chlorpromazine neutralized the effect of dopamine for all strains in a concentration-dependent way (**Figure 22A**). The β-adrenergic antagonist propanolol had a very small effect on norepinephrine-induced swimming motility of the *V. anguillarum* strains, whereas it had no effect on norepinephrine-induced motility of the *V. campbellii* strains (**Figure 22B**). In contrast, the α-adrenergic receptor antagonists phenoxybenzamine and phentolamine significantly blocked norepinephrine-induced motility of all tested strains, although the effect was modest for phenoxybenzamine in *V. campbellii* LMG 21363 and *V. anguillarum* NB10 (**Figure 22C** and **Figure 22D**). Finally, neither dopamine nor norepinephrine affected hemolysin, protease and chitinase activities in any of the tested strains (data not shown).

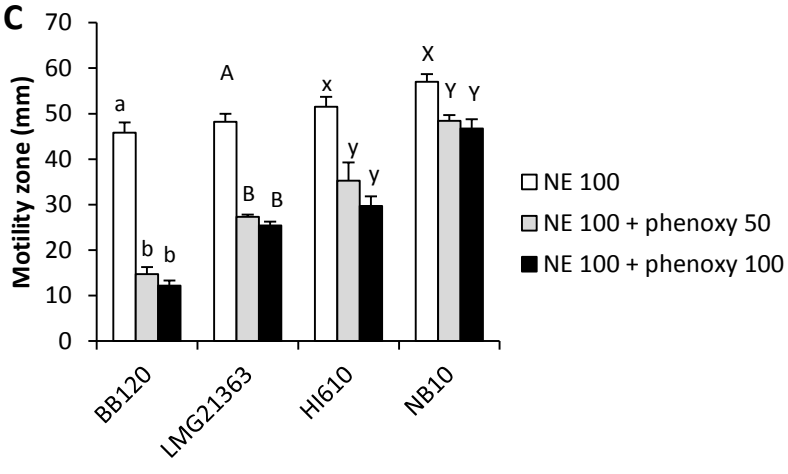
Impact of norepinephrine and dopamine on growth of the vibrios in the presence of serum

The impact of catecholamines on growth of bacteria thus far mostly has been investigated in SAPI medium with serum. However, none of the vibrios was able to grow in SAPI, even without serum (data not shown). Therefore, we decided to study the effect in LB_{12} medium, with and without the addition of serum. The addition of serum resulted in a complete growth inhibition in LB_{12} for all strains tested in the absence of catecholamines. **Figure 23A** and **23C**, and **Figure 24A** and **24C** show the results obtained for strains BB120 and NB10 as examples for *V. campbellii* and *V. anguillarum*, respectively. In the absence of serum, the catecholamines had no effect on growth (**Figure 23B** and **23D**, and **Figure 24B** and **24D**).

We further investigated whether eukaryotic catecholamine receptor antagonists could neutralize the impact of the catecholamines on growth in the presence of serum. The dopaminergic receptor antagonist chlorpromazine and the adrenergic receptor antagonist phentolamine were selected based on their effect in the motility assays. The antagonists were not able to significantly neutralize the effect of dopamine and norepinephrine when the hormones were added at 100 µM (data not shown). However, the antagonists (at 100 µM) were able to (partially) inhibit catecholamine-induced growth obtained at 50 µM of the catecholamines (**Figure 23A** and **23C** and **Figure 24A** and **24C**). The receptor antagonists did not affect the growth of the vibrios in LB_{12} medium without serum (**Figure 23B** and **23D** and **Figure 24B** and **24D**).


A
Motility zone (mm)
60
50
40
30
20
10
0
a
b
c
A
B
C
x
y
z
X
Y
Z
BB120
LMG21363
HI610
NB10
Dopa 100
Dopa 100 + chlor 50
Dopa 100 + chlor100


B
Motility zone (mm)
80
70
60
50
40
30
20
10
0
a a a
A A A
x xy y
X XY Y
BB120
LMG21363
HI610
NB10
NE 100
NE 100 + propanolol 50
NE 100 + propanolol 100


C
Motility zone (mm)
70
60
50
40
30
20
10
0
a
b b
A
B B
x
y y
X
Y Y
BB120
LMG21363
HI610
NB10
NE 100
NE 100 + phenoxy 50
NE 100 + phenoxy 100

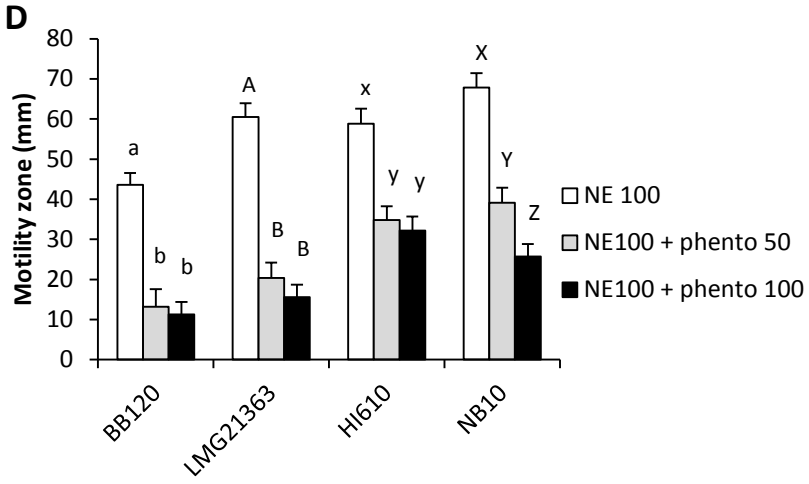

D
Motility zone (mm)
80
70
60
50
40
30
20
10
0
a
b b
A
B B
x
y y
X
Y
Z
BB120
LMG21363
HI610
NB10
NE 100
NE100 + phento 50
NE100 + phento 100

Figure 22. The impact of eukaryotic catecholamine receptor inhibitors on catecholamine-induced swimming motility in the aquaculture pathogens *V. campbellii* BB120, *V. campbellii* LMG 21363, *V. anguillarum* HI610 and *V. anguillarum* NB10 after 24h incubation on soft agar. **(A)** Dopamine (Dopa; 100 µM) with or without the dopaminergic antagonist chlorpromazine (chlor) at 50 µM or 100 µM, **(B)** Norepinephrine (NE; 100 µM) with or without the β-adrenergic antagonist propanolol at 50 µM or 100 µM, **(C)** Norepinephrine with or without the α-adrenergic antagonist phenoxybenzamine (phenoxy) at 50 µM or 100 µM, **(D)** Norepinephrine with or without the α-adrenergic antagonist phentolamine (phento) at 50 µM or 100 µM. Error bars represent the standard deviation of five replicates. For each panel, bars with a different letter are significantly different from each other (ANOVA with Tukey's post-hoc test; $p < 0.01$).

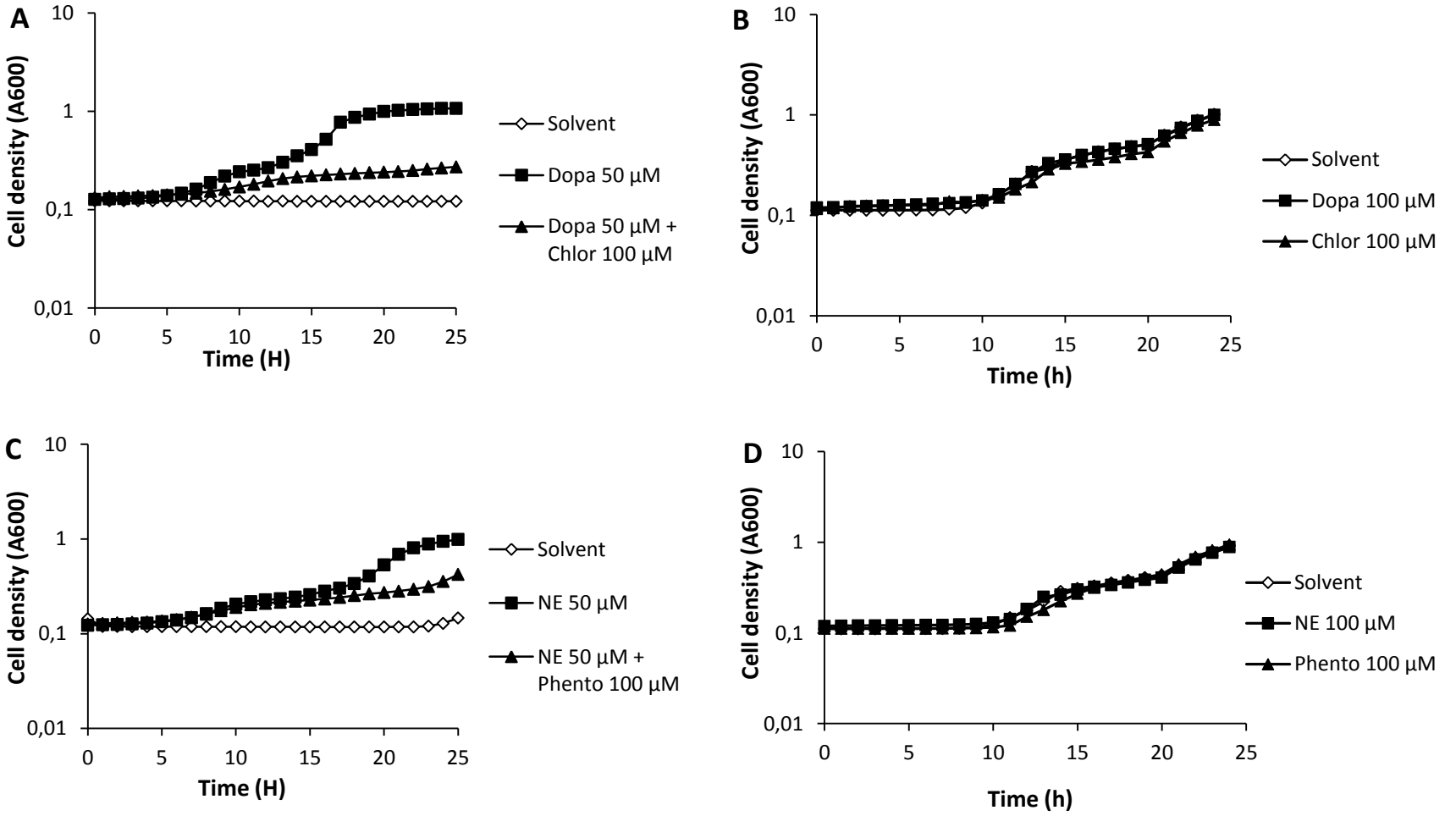


Figure 23. Growth of *V. campbellii* BB120 in LB_{12} broth, with and without the catecholamines dopamine (Dopa) and norepinephrine (NE) and the catecholamine receptor antagonists chlorpromazine (Chlor) and phentolamine (Phento). **(A)(C)** LB_{12} broth with serum. **(B)(D)** LB_{12} broth without serum. Error bars showing the standard deviation of three replicates are too small to be visible. . "Solvent" refers to *V. campbellii* treated with the same volume of solvent as added with the catecholamines and/or antagonists (in case they were dissolved in organic solvents).

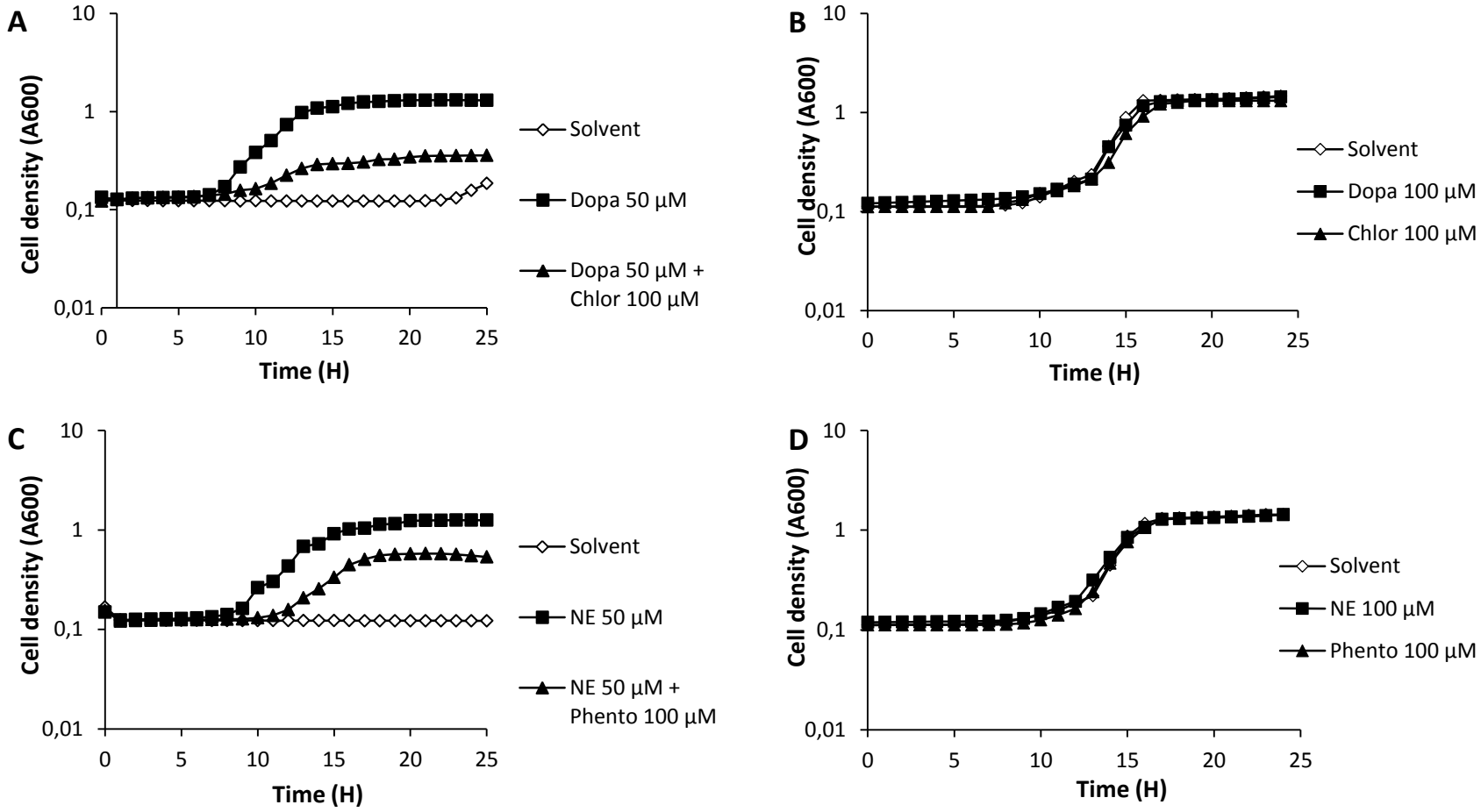


Figure 24. Growth of *V. anguillarum* NB10 in LB_{12} broth, with and without the catecholamines dopamine (Dopa) and norepinephrine (NE) and the catecholamine receptor antagonists chlorpromazine (Chlor) and phentolamine (Phento). **(A)(C)** LB_{12} broth with serum. **(B)(D)** LB_{12} broth without serum. Error bars showing the standard deviation of three replicates are too small to be visible. . "Solvent" refers to *V. campbellii* treated with the same volume of solvent as added with the catecholamines and/or antagonists (in case they were dissolved in organic solvents).

Impact of catecholamines and catecholamine receptor antagonists on the virulence of *Vibrio campbellii* BB120 towards giant freshwater prawn larvae

We investigated the impact of the catecholamines and their respective receptor antagonists on the virulence of *V. campbellii* BB120 towards giant freshwater prawn larvae. Chlorpromazine was used as dopaminergic receptor antagonist and phentolamine was selected as adrenergic receptor antagonist. In order to avoid any direct effect of the catecholamines on the host, *V. campbellii* was pretreated with the catecholamines (with or without antagonist), after which the compounds were removed prior to inoculation into the rearing water of the larvae. The catecholamines significantly increased the virulence of *V. campbellii* (**Table 13**).

Table 13. Percentage survival of *M. rosenbergii* larvae after challenge with *V. campbellii* BB120 with and without catecholamines and inhibitors (average ± standard deviation of five replicates). Experiment I, II and III were conducted for 8, 8 and 6 days, respectively.

Treatments	Survival (%)		
	Experiment I	Experiment II	Experiment III
Control	70 ± 10^{c}	72 ± 6^{c}	76 ± 6^{d}
BB120	50 ± 5^{b}	50 ± 5^{b}	49 ± 5^{bc}
BB120 + NE 100 µM	36 ± 6^{a}	33 ± 5^{a}	32 ± 5^{a}
BB120 + NE 100 µM + Phentolamine 200 µM	49 ± 7^{ab}	34 ± 6^{a}	49 ± 3^{bc}
BB120 + NE 100 µM + Phentolamine 500 µM	52 ± 3^{b}	31 ± 7^{a}	58 ± 2^{c}
BB120 + Dopa 100 µM	36 ± 5^{a}	33 ± 5^{a}	38 ± 2^{ab}
BB120 + Dopa 100 µM + Chlorpromazine 50 µM	38 ± 8^{a}	34 ± 7^{a}	49 ± 2^{bc}
BB120 + Dopa 100 µM + Chlorpromazine 100 µM	39 ± 8^{a}	36 ± 4^{ab}	55 ± 3^{c}

Values in the same coloumn with different superscript letters are significantly different (p<0.01)
NE: norepinephrine; Dopa: dopamine

Further, the dopaminergic receptor antagonist chlorpromazine could not neutralize the effect of dopamine, whereas the adrenergic receptor antagonist phentolamine only neutralized the effect of norepinephrine at a relatively high concentration and in only 2

of the 3 trials. Finally, although there were significant differences in survival between the different treatments, there were no differences in growth of the surviving larvae (data not shown).

DISCUSSION

Vibrio spp. are virulent pathogens in fish and shellfish aquaculture worldwide. Disease outbreaks in aquaculture may occur when environmental factors trigger the rapid multiplication of pathogens (De Schryver et al., 2014). Other factors are stressful conditions which evoke neuroendocrine responses of the hosts involving the release of catecholamines (Li et al., 2005). In the current study, we demonstrated that the catecholamines norepinephrine and dopamine significantly induced the swimming motility of *V. anguillarum* and *V. campbellii*. Swimming motility is an important phenotype with respect to infection of both *V. campbellii* and *V. anguillarum* and is probably involved in host colonization (O'Toole et al., 1996; Yang & Defoirdt, 2014). Our finding is in agreement with previous work in which catecholamines were found to enhance the motility of *E. coli* (Kendall et al., 2007), *Salmonella typhimurium* (Bearson & Bearson, 2008), *Campylobacter jejuni* (Cogan et al., 2007) and the fish pathogen *Edwardsiella tarda* (Wang et al., 2011).

Catecholamines are produced by hemocytes of invertebrates and have been reported to be present in the eyestalk, thoracic ganglion and hemolymph of giant freshwater prawn. Concentrations in the hemolymph of shrimp and prawn that have been reported range between 10 nM and ~3 µM (Chen et al., 2003; Hsieh et al., 2006; Pan et al., 2014). These concentrations are lower than the concentrations used in this study (100 µM). However, local concentrations can be considerably higher than those that are found in hemolyph (or in serum in case of vertebrates). For instance, the intrasynaptic concentration of norepinephrine in the central nervous system of mammals is as high as 10 mM (vs. nM levels in serum) (Lyte, 2004). Hence, upon infection, pathogens can come into contact with local concentrations that are several orders of magnitude higher than those that

are found in hemolymph or blood. This probably is also the case when tissues and/or hemocytes are damaged during infection.

We further found that chlorpromazine, an antagonist of eukaryotic dopamine receptors, could neutralize the motility-inducing effect of dopamine. Meanwhile, the α-adrenergic receptor antagonists phentolamine and phenoxybenzamine could neutralize the motility-inducing effect of norepinephrine, whereas the β-adrenergic receptor antagonist propranolol had no or very little effect. This is in agreement with a previous study showing that α-adrenergic receptor antagonists neutralize the motility inducing-effect of norepinephrine in intestinal pathogens (Freestone et al., 2007). In contrast, other reports showed that both α-adrenergic and β-adrenergic receptor antagonists were able to block the response of *E. coli* O157:H7 to norepinephrine and epinephrine (Sperandio et al., 2003).

Norepinephrine and dopamine enabled all tested *Vibrio* strains to grow in the presence of serum (which is added to mimic the host environment where the availability of iron is also limited by high affinity ferric iron binding proteins such as transferrin (Freestone et al., 2008)), and the addition of antagonists of eukaryotic catecholamine receptors together with the catecholamines could partially neutralize this effect. Catecholamines have been shown to increase the growth of different Gram-negative bacteria (including *Vibrio parahaemolyticus* and *Vibrio mimicus*) in minimal medium containing serum (Lyte, 2004; Nakano et al., 2007b). Iron is important with respect to proliferation of bacterial pathogens during infection and its acquisition from the iron-binding protein transferrin is critical for growth when cultured in the presence of serum (Freestone et al., 2003). The mechanistic explanation that prevails in literature is that catecholamines form complexes with transferrin, which reduces the ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}), a valency for which iron binding proteins have a much lower affinity. The complex formation thus results in weakening of the bond between iron and transferrin (Sharaff & Freestone, 2011; Freestone, 2013). Our observation that catecholamine receptor antagonists are able to partially neutralize the growth-stimulatory effect of catecholamines suggests that a regulatory mechanism involving catecholamine

receptors is involved as well, and this has also been reported for enteric pathogens such as *E. coli* O157:H7, *Salmonella enterica* and *Yersinia enterocolitica* (Freestone et al., 2007). Finally, it should be noted that in addition to limiting iron availability, serum might have other effects on the pathogens as well.

Our *in vivo* challenge tests revealed that pretreatment of *V. campbellii* with catecholamines increased the virulence of the bacterium towards giant freshwater prawn larvae, whereas the growth of the surviving larvae was not influenced. By pretreating the pathogen with catecholamines, we avoided any direct effect of the compound on the host (e.g. decreased activity of the defense system), ensuring that any impact on survival of challenged prawn larvae was due to increased virulence of the pathogen. *V. campbellii* was pretreated with catecholamines to simulate transmission from a host site where catecholamines were present (e.g. due to stress and/or cell and tissue damage). In combination with our observation that the catecholamines increased swimming motility of the vibrios (an important factor during the initial stages of infection; McCarter (2004)), our results indicate that sensing elevated catecholamine levels increases the success of transmission to a new host. Hence, elevated catecholamine levels might be a cue informing the pathogen that the infection reached a final stage (cell and tissue damage) and that it is time to leave the host.

In contrast to our *in vitro* data showing that catecholamine receptor antagonists could neutralize the effects of the catecholamines, the effect of the antagonists towards *in vivo* virulence was not consistent, with phentolamine having an effect in 2/3 independent experiments and chlorpromazine having an effect in only 1/3 experiments. One explanation might be that differences in the activities of the larvae-associated microbiota (e.g. production of compounds that interfere with catecholamine sensing) and/or the response of the larvae to the microbiota (e.g. by producing different levels of catecholamines) might be responsible for the differences between the trials. Another explanation might be that in addition to the stimulation of growth and virulence via a receptor-mediated mechanism (which is affected by the antagonists), the catecholamines also stimulate virulence through another mechanism not involving these

receptors. One such mechanism could be the induction of a stimulator of growth, as described before in enteric bacteria (Lyte et al., 1996; Freestone et al., 1999).

In conclusion, our study showed that catecholamines can increase the virulence of aquaculture pathogenic vibrios by increasing their growth in an iron-limited environment and by increasing swimming motility. As swimming motility is required during the early stages of infection, the catecholamine-induced increase in swimming motility can be an important factor to increase the success of transmission to a new host. Catecholamine-induced effects could be neutralized by antagonists for eukaryotic catecholamine receptors, suggesting that receptors with a similar specificity are involved in the response of the vibrios to the catecholamines.

ACKNOWLEDGEMENTS

This work was funded by the Research Foundation of Flanders (FWO-Vlaanderen; project n^{o} 1.5.013.12N), by the Belgian Science Policy Office (Belspo IUAP project No IUAP/64/Sorgeloos) and by the Directorate General of Higher Education of Indonesia through a doctoral scholarship to Pande Gde Sasmita Julyantoro. Tom Defoirdt is a postdoctoral fellow of FWO-Vlaanderen. The authors have no conflicts of interest to declare.

CHAPTER 7

GENERAL DISCUSSION

INTRODUCTION

As an alternative to antibiotics, interference with pathogen-pathogen and host pathogen signaling have been proposed as a new method to combat bacterial diseases in aquaculture. This study addresses the impact of bacteria-bacteria and host bacteria signaling on vibriosis in larviculture of giant freshwater prawn *Macrobrachium rosenbergii*. The results showed that bacterial cell-to-cell communication or quorum sensing regulates the virulence of *Vibrio campbellii* toward giant freshwater prawn larvae. Furthermore, quorum sensing disrupting compounds and quorum sensing molecule-degrading bacteria were found to be able to protect the animals from the pathogen. With respect to host factors, we found that catecholamines stress hormones produced by the host are able to increase the virulence of bacterial aquaculture pathogens. Although eukaryotic catecholamine receptor antagonists could neutralize the effect of the respective catecholamines *in vitro*, the effects of the receptor antagonist were less clear *in vivo*. In this chapter, the most important findings of this work are highlighted and put in perspective, and suggestions for future research are presented.

EFFECT OF DIFFERENT QUORUM SENSING SIGNAL MOLECULES ON THE VIRULENCE OF *VIBRIO CAMPBELLII* TOWARDS DIFFERENT HOSTS

Vibriosis is one of the most critical diseases in shrimp aquaculture. It can cause 100% mortality in early life stages of shrimp, such as larvae, postlarvae and juveniles (Austin & Zhang, 2006). *Vibrio* disease is also described as penaeid bacterial septicemia, penaeid vibriosis, luminescent vibriosis or red-leg disease (Ruwandeepika et al., 2012). Luminescent vibriosis caused by *Vibrio campbellii* and closely related species has been reported to cause huge losses and is a major constraint to shrimp production especially in South America and Asia (Austin & Zhang, 2006). Virulence factor production in bacterial pathogens, including *Vibrio* species, is under a strict regulatory control, including quorum sensing and sensing of hosts factors (Ruwandeepika et al., 2012).

In **Chapter 3**, we examined the impact of the three different quorum sensing signals produced by *Vibrio campbellii* on the virulence of the bacterium towards giant

freshwater prawn larvae. Quorum sensing is one of the regulatory mechanisms by which bacteria coordinate the expression of certain genes in response to the presence of small signal molecules, enabling bacteria to interact with each other and with their environment (Hense et al., 2007). However, studies on the impact of quorum sensing on the *in vivo* virulence of the pathogens toward aquatic organisms are very limited. We found that although they use a shared signal transduction cascade, the signal molecules have a different impact on the virulence of the bacterium towards different hosts. Previous research showed that the quorum sensing signal molecules AI-2 and CAI-1 are needed for full virulence of the pathogen towards brine shrimp larvae (*Artemia franciscana*), whereas HAI-1 had no effect (Defoirdt et al., 2005; Defoirdt and Sorgeloos, 2012). In contrast, the present study showed that HAI-1 and AI-2 are needed for full virulence towards commercially important giant freshwater prawn (*Macrobrachium rosenbergii*), whereas CAI-1 had no effect in this host.

The different pH of the host environment (i.e. the site where the pathogens are associated with the host) might influence the stability of the signal molecules. The pH in the brine shrimp gastrointestinal tract might be similar to that of seawater, whereas prawn larvae are larger, which might enable them to maintain an internal pH that is lower than the surrounding environment. Indeed, the alkaline hydrolysis of the lactone ring of HAI-1 at pH >8 has been shown to result in a low stability of HAI-1 in the marine environment (Byers et al., 2002; Yates et al., 2002), therefore HAI-1 might be inactive in brine shrimp culture. However, there is little supporting information about the intestinal pH of these hosts. A deep investigation on the effect of different pH on the quorum sensing signal molecule activity *in vitro* and *in vivo* might be an interesting topic for further research.

The ability of the eukaryotic host to produce quorum sensing signal-inactivating enzymes, for example AHL degradation activity due to the paraoxonases encoded by the *PON* genes in mammals (Greenberg et al., 2004), might also be a possible explanation, as reported by Dong and co-workers (2007). Other possibilities are that the expression of signal molecule synthases and/or receptors might be different in different environments

or there might be as yet non-identified signal transduction cascades that are affected by only one or two of the three signals.

Quorum sensing systems have been reported to regulate the expression of virulence genes in many pathogenic bacteria (**Table 14**), and many of them use several signal molecules to regulate virulence gene expression (Jayaraman & Wood, 2008; Ng & Bassler, 2009). In aquaculture pathogens, three channel quorum sensing systems have also been identified or predicted in *Vibrio anguillarum* and *Vibrio parahaemolyticus*. AHLs and AI-2 have been discovered in *Vibrio vulnificus*, *Vibrio icthyoenteri*, *Aeromonas hydrophila*, and *Edwardsiella tarda*. AI-2 and CAI-1 have been reported in *Vibrio alginolyticus*. AI-2 has been reported in *Vibrio mimicus*, and AHLs have been reported in *Allivibrio salmonicida*, *Aeromonas salmonicida*, *Yersinia ruckeri* and *Tenacibaculum maritimum* (Zhao et al., 2014). However, it is important to take into consideration that quorum sensing regulation of a particular virulence factor may differ for different bacterial species, as in addition to quorum sensing the expression of virulence genes is regulated by other factors as well, e.g. host factors (Natrah, 2011; Li et al., 2015). Some host factors including mucus have been reported to enhance growth (Garcia et al. 1997), protease activity (Denkin & Nelson 1999) and chemotactic motility in *Vibrio anguillarum* (Larsen et al., 2001). Bile and bile salts have been reported to affect virulence gene expression in *Vibrio cholerae* (Yang et al., 2013) and *Vibrio parahaemolyticus* (Pace et al., 1997; Osawa et al., 2002; Gotoh et al., 2010). Cholesterol has been reported to affect the production of virulence factors in *Vibrio vulnificus* (Kim & Kim 2002) and *Vibrio cholerae* (Chatterjee et al., 2007).

Tabel 14. Impact of quorum sensing on the virulence of different bacterial aquaculture pathogens towards different aquatic hosts.

Pathogen	Host	Impact of QS signaling on virulence	References
Aeromonas hydrophila	Burbot larvae (*Lota lota*)	BHL required for full virulence	Natrah et al., 2012
	Zebrafish (*Danio rerio*)	BHL required for full virulence	Chu et al., 2012
	Common carp (*Cyprinus carpio*)	AhyR mutant has decreased virulence	Bi et al., 2007
Aeromonas salmonicida	Burbot larvae (*Lota lota*)	BHL required for full virulence	Natrah et al., 2012
Aeromonas salmonicida Subsp. *achromogenes*	Archtic carr juveniles (*Salvelinus alvinus* L.)	BHL required for full virulence	Schwenteit et al., 2011
Edwardsiella tarda	Zebrafish (*Danio rerio*)	EdwR had no direct role in virulence toward zebrafish	Han, 2011
Vibrio alginolyticus	Red sea bream juveniles (*Pagrus major*)	Inactivation of *luxS* decreased the virulence	Ye at al., 2008
	Zebrafish (*Danio rerio*)	Inactivation of luxT slightly decreased the virulence	Liu et al., 2012
Vibrio anguillarum	Sea bass (*Dicentrarchus labrax*) larvae	Increased levels of indole decreased the virulence	Li et al., 2014
	Rainbow trout (*Oncorhynchus mykiss*)	Three-channel QS system, VanI and VanR have no impact on virulence	Milton et al., 1997
Vibrio campbellii	Brine shrimp (*Artemia franciscana*)	AI-2 and CAI-1 required for full virulence	Defoirdt, 2007
or	Rotifer (*Brachionus plicatilis*)	HAI-1 or AI-2 required for virulence	Tinh et al., 2007
Vibrio harveyi	Giant freshwater prawn (*Macrobrachium rosenbergii*) larvae	HAI-1 and AI-2 required for full virulence	This study
Vibrio ichthyoenteri	Turbot (*Scopthalmus maximus*)	LuxS mutant is still virulent	Li et al., 2010

AhyR : *Aeromonas hydrophila* LuxR homologue, EdwR : *Edwardsiela tarda* LuxR homologue, LuxS : AI-2 synthase protein
LuxT : protein regulator that play multiple roles in regulating extracellular proteases production and motility in *Vibrio alginolyticus*.

The regulatory networks controlling virulence are probably much more complicated than what is currently known. For instance, previously, *Pseudomonas aeruginosa* had been reported to regulate the production of numerous virulence factors via the action of two separate but coordinated quorum sensing systems, *las* and *rhl*. These systems control the transcription of genes in response to population density through the intercellular signals N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C(12)-HSL) and N-(butanoyl)-L-homoserine lactone (C(4)-HSL). Later on, a third *Pseudomonas aeruginosa* signal, 2-heptyl-3-hydroxy-4-quinolone [*Pseudomonas* quinolone signal (PQS)] was also found to play a significant role in the transcription of multiple *Pseudomonas aeruginosa* virulence genes (McGrath et al., 2004). Currently, a much more complex regulatory network including 650 genes organized into 11 hierarchies, encompassing important biological processes, such as biofilms formation, production of the exopolysaccharide alginate and several virulence factors, and of the quorum sensing regulons have been documented in *Pseudomonas aeruginosa* (Galan-Vasquez et al., 2011).

Focusing on the *Vibrio campbellii* quoum sensing system, although the three channels are connected to LuxO as a central protein in the signal transduction cascade, the expression of signal molecule synthases and/or receptors might be different in different environments. Moreover, the regulation of different quorum sensing genes controlled by the master regulator LuxR is different for different genes since different target promoters have varying affinities for LuxR (Waters & Bassler, 2007).

An interesting report from Teng & co-workers (2011) showed that *Vibrio campbellii* can alter the ratio between the signal molecule receptors, allowing cells to pay more attention to one of the signals under certain conditions. The receptor ratios are simultaneously controlled through multiple feedback loops thus ensuring fidelity (Natrah, 2011). *Vibrio campbellii* virulence being controlled by only two out of the three signals might be due to the fact that these signals resulted in a sufficiently high level of LuxR to activate virulence gene expression. Alternatively, there might be additional (yet not unraveled) signal transduction cascades that are affected by only one or two of the

signal molecules. Indeed, another interesting research from Ruwandeepika and co-workers (2011) found that the expression of the *vhh* haemolysin gene is not regulated by the three-channel quorum sensing system, whereas a lower expression was observed in the AI-2 production and detection mutants. This suggested that an additional signal transduction cascade that is only affected by AI-2 exists and that the AI-2 signal molecule controls *vhh* expression using a pathway that is distinct from the LuxO pathway.

QUORUM SENSING INHIBITORS PROTECT AQUACULTURE ANIMALS FROM VIBRIOSIS

The adverse effects related to the inappropriate use of antibiotics in aquaculture are forcing researchers to develop alternative methods to control disease. Many recent studies showed that many pathogenic bacteria regulate their virulence through quorum sensing, therefore the inhibition of quorum sensing system has been proposed as a novel biocontrol strategy. Quorum sensing inhibition in aquaculture pathogenic bacteria can be implemented by different methods (Defoirdt et al., 2004). It is highly recommended to study the efficacy of quorum sensing inhibitors as novel biocontrol agents under conditions that are as close as possible to the clinical situation. In this study, two different methods to disrupt *Vibrio campbellii* quorum sensing have been explored by *in vivo* challenge test toward prawn larvae by using quorum sensing-disrupting compounds and quorum sensing signal molecule-degrading bacteria.

In **Chapter 4**, the capabilities of three quorum sensing-disrupting compounds to protect prawn larvae from *Vibrio campbellii* were evaluated. These compounds were cinnamaldehyde, the brominated furanone ((*Z*-)-4-bromo-5-(bromomethylene)-2(*5H*)-furanone) and the brominated thiophenone ((*Z*)-4-((5-(bromomethylene)-2-oxo-2,5-dihydrothiophen-3-yl)methoxy)-4-oxobutanoic acid). All three of these compounds had been shown before to disrupt quorum sensing in vibrios by decreasing the DNA-binding activity of the quorum sensing master regulator LuxR (Defoirdt et al., 2007b; Brackman et al., 2008; Defoirdt et al., 2012). We found that addition of 1 µM of these compounds to the rearing water resulted in a complete protection of challenged larvae, without any

impact on the growth of surviving larvae. However, increasing the concentration of the compounds to 10 µM did not further increase the survival of the larvae and 10 µM of furanone even resulted in complete mortality (Pande et al., 2013b).

Previously, cinnamaldehyde has been reported to protect brine shrimp larvae from pathogenic *Vibrio campbellii* (Brackman et al., 2008) and also to protect burbot (*Lota lota* L.) larvae from *Aeromonas hydrophila* and *Aeromonas salmonicida* (Natrah et al., 2012). Meanwhile, brominated furanones have been reported before to protect brine shrimp (*Artemia franciscana*) larvae from pathogenic *Vibrio campbellii* (Defoirdt et al., 2006a) and rainbow trout (*Oncorhyncus mykiss*) from *Vibrio anguillarum* (Rasch et al., 2004), and the thiophenone compound has also been reported before to protect brine shrimp larvae from *Vibrio campbellii* (Defoirdt et al., 2012).

Potential side effects of these quorum sensing-disrupting compounds should be assessed before applying them to real aquaculture facilities. Indeed, some compounds could be toxic to the reared animals. In our study, 10 µM of furanone resulted in complete mortality of the prawn larvae. It is consistent with the literature reporting that these furanones show considerable toxicity towards higher organisms (Hentzer and Givskov, 2003; Defoirdt et al., 2006a). This compound also caused complete mortality of Burbot (*Lota lota*) larvae when applied at 0, 01 µM (Natrah et al., 2012). In brine shrimp larvae, the used dosages were relatively high (up to 80 µM) (Defoirdt et al., 2006), whereas cinnamaldehyde was used at 100 µM (Brackman et al., 2009). Apparently, giant freshwater prawn larvae are less tolerant to the furanone than brine shrimp larvae. Also rotifers (*Brachionus plicatilis*) are able to survive higher concentration (10 µM) (Tinh et al., 2007). The brominated thiophenone has similar activity as the furanone but is less toxic to the prawn larvae. In addition to potential negative effects to the reared animals, the impact of these compounds on the environment should be assessed as well before they can be used in practice. In the natural environment, quorum sensing-disrupting compounds might have a negative effect on harmless bacteria that also use quorum sensing mechanisms to regulate beneficial processes such as symbiosis, sporulation and

biofilm formation in aquaculture recirculation system (Miller & Bassler, 2001; Defoirdt et al., 2004, Natrah, 2011).

Chapter 5 focused on quorum sensing disruption by acyl-homeserine lactone (AHL)-degrading bacteria isolated from microalgal cultures. Microalgae are an important constituent in many aquaculture systems, especially in the so-called green-water systems, which are characterised by high levels of microalgae in the rearing water (Hargreaves, 2006; Palmer et al., 2007). These green-water systems are currently used to culture various aquaculture animals (FAO, 2013) and empirical evidence points to a better growth and survival in these systems when compared to the conventional clear water aquaculture (Muller-Feuga, 2000). However, the mechanisms by which green-water exert a beneficial effect are still poorly understood, especially the potential beneficial effects of bacteria associated with micro-algae remain largely unexplored (Natrah et al., 2013). The bacteria associated with micro-algae might also have an important role in protecting the reared animals from disease. Degradation of quorum sensing signal molecules has been proposed to reduce the virulence of pathogens, and this ability is widely distributed in the bacterial kingdom (Dong et al., 2007; LaSarre & Federle, 2013). Because the concentration of an AHL signal is a key factor in mediating virulence gene expression, it was reasoned that a strategy could be developed for the control of bacterial infection by degrading the AHL signals produced by pathogenic bacteria (Dong et al., 2000).

Our study confirmed that two isolates, *Bacillus* sp. NFMI-C and *Pseudomonas* sp. NFMI-T isolated from laboratory cultures of *Chaetocheros muelleri* and *Tetraselmis suecica*, respectively, were able to degrade *N*-(hexanoyl)-L-homoserine lactone within 10 hours and 3 hours when growing as axenic culture and mixture culture, respectively, in a nutrient-rich background. However, we found that only *Bacillus* sp. NFMI-C, but not *Pseudomonas* sp. NFMI-T, was able to inactivate the AHL produced by V*ibrio campbellii* as determined by measuring quorum sensing-regulated bioluminescence.

The AHL molecules produced by different bacteria all shares the homoserine lactone moiety, but they differ in the length and substitution of the acyl side chain (Dong & Zhang, 2005). The structural features of AHLs suggest that there may be at least four types of enzymes that could degrade AHL signals (Dong & Zhang, 2005), Among them, lactonases and decarboxylases hydrolyze the lactone ring at the positions marked as 1 and 2, while acylases and deaminases separate the homoserine lactone moiety and acyl side chain at sites 3 and 4, respectively (**Figure 25**).

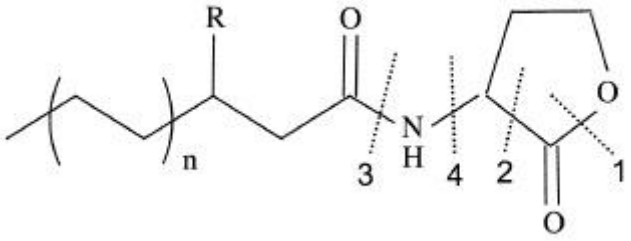


Figure 25. AHL structure and its potential cleavage sites by AHL inactivating enzymes

To date, only two groups of AHL-degrading enzymes have been demonstrated enzymatically and structurally to inactivate AHL substrates, i.e., acyl-homoserine lactone lactonases and acyl-homoserine lactone acylases (Dong et al., 2001; Zhang et al., 2002; Lin et al., 2003; Park et al., 2003). Several *Bacillus* species have been reported to produce AHL lactonases, which inactivate AHLs by hydrolysing the lactone ring (Dong & Zhang, 2005). Lactonases are intracellular enzymes capable of inactivating a wide range of AHLs, varying in acyl chain length and substitution (Dong et al., 2007). Meanwhile, *Pseudomonas* species have been reported to produce AHL acylases, which cleave AHLs by aminohydrolysis into homoserine lactone and fatty acid (Dong & Zhang, 2005). Unlike lactonases, acylases exhibit substrate specificity depending on the acyl side chain length and the substitution on the β-position of the acyl chain (Tang & Zhang, 2014). This might explain why *Pseudomonas* sp. NFMI-T was not able to interfere with quorum sensing in *V. campbellii* although it was able to degrade HHL. Finally, addition of *Bacillus* sp. NFMI-C to the rearing water of giant freshwater prawn (*Macrobrachium rosenbergii*) larvae significantly improved survival of the larvae when challenged with pathogenic *Vibrio campbellii,* whereas it had no effect on larval growth.

Generally, the application of *Bacillus* sp. in aquaculture is growing rapidly, especially for intensive aquaculture systems (Hong et al., 2005). Some advantages of using *Bacillus* spp. in aquaculture are the ability of this genus to replicate rapidly, tolerate a multitude of environmental conditions and provide a broad range of beneficial effects that can improve aquaculture productivity (Lalloo et al., 2009). Importantly, *Bacillus* sp. are not generally involved in horizontal gene transfer processes with aquaculture pathogenic bacteria such as *Vibrio* and *Aeromonas* spp. and are thus unlikely to acquire genes for antibiotic resistance (Moriarty, 1998).

Bacillus spp. are commonly used as components of biocontrol products which are often composed of mixtures of species, which are able to exert a range of beneficial effects on aquaculture systems (Lalloo et al., 2007). This genus has an ability to form endospores which are rigid structures that are capable of surviving under harsh conditions. The spores can be used as biological agents due to the many advantages of this form over vegetative cells, because of their higher resistance to external factors such as mechanical force, desiccation, solar radiation and high temperatures (Wolken et al., 2003). Products containing spores can be stored in a stable form for long periods under challenging conditions normally prevalent on aquaculture farms (Hong et al., 2005).

Several studies have demonstrated the application of *Bacillus* sp. in aquaculture, such as fed to turbot larvae resulted in a decrease of *Vibrionaceae* population with significant improvement in weight gain and survival of the larvae (Gatesoupe, 1999), improved food absorption by enhancing protease levels, and improved turbot larval growth (Irianto & Austin, 2002). It was also reported that *Bacillus* sp. had significantly reduced development times of *Penaeus monodon* larvae and protect them from pathogenic *V. harveyi* infection (Rengpipat et al., 2000). Furthermore, the use of *Bacillus* sp. in several penaeid culture ponds in Indonesia enhanced the production of shrimps by preventing bacterial disease caused by luminescent *Vibrio* spp. (Moriarty, 1998). Finally, the biosafety and licensing issues that affect the use of *Bacillus* species for commercial development should be evaluated on case by case to exclude the possibility that it belongs to a species containing pathogenic strains (Hong et al., 2005).

THE ROLE OF CATECHOLAMINE SENSING ON THE VIRULENCE OF BACTERIAL AQUACULTURE PATHOGENS

Host factors are known to affect virulence in various pathogenic bacteria in general and vibrios more specifically (Li, 2014). The physiological condition of the host (such as stress) and some metabolic products have a significant impact on the success of bacterial infection. In stressful conditions, neuroendocrine responses of the host are evoked including the release of stress hormones, such as catecholamines (Li et al., 2005). These mechanisms are conserved in vertebrates and invertebrates including aquaculture animals such as fish and shrimp (Ottaviani & Franceschi, 1996). The development of microbial endocrinology as new microbiological research field followed from the discovery that microorganisms have evolved systems for sensing hormones produced by the host (Freestone, 2013). To date the majority of investigations have focused on the interaction of bacteria with the stress-associated catecholamines adrenaline, noradrenaline (noerepinephrine) and dopamine (Lyte, 2004, Freestone et al., 2008).

Catecholamines are derived from tyrosine and characterized by having a catecholate moiety linked to an amine side chain (Defoirdt, 2013b). Adrenaline and noradrenaline are the integral part of the acute 'fight and flight' stress response in higher animals and conserved among vertebrates and invertebrates (Ottaviani & Franceschi, 1996). In mammals, the nerve terminals that contain norepinephrine and dopamine are distributed throughout the body, including the intestinal tract, where they are part of enteric nervous system (Defoirdt, 2013b). More than 50% of the total amount of noradrenaline in the mammalian body is produced by the mesenteric organs (Lyte, 2004). Meanwhile, in invertebrates the stress response is concentrated in the haemocyte (Ottaviani & Franceschi, 1996).

In **Chapter 6**, we investigated the impact of catecholamine stress hormones (norepinephrine and dopamine) on growth and virulence factor production in pathogenic vibrios (i.e. 2 *Vibrio campbellii* strains and 2 *Vibrio anguillarum* strains). Both

norepinephrine and dopamine (at 100 µM) significantly induced growth in serum-based media and also increased swimming motility of the tested strains, whereas they had no effect on caseinase, chitinase and hemolysin activities *in vitro*. So far, the effect of catecholamines on bacterial infection has been studied *in vitro* by investigating impact on growth, virulence and gene expression with dosage range of 50 µM – 5 mM (Lyte & Freestone, 2010) in serum-based media (to mimic the bacteriostatic environment inside the host through the sequestration of free Fe by high affinity ferric iron binding proteins such as transferrin and lactoferrin) (Freestone & Lyte, 2008; Ratledge & Dover, 2000). The mechanism by which catecholamines induce growth in these media has been described in the discussion section of **Chapter 6**, i.e. catecholamines enable bacteria to access the sequestered Fe within the host (Freestone et al., 2007, Sandrini et al., 2010). Swimming motility is an important phenotype with respect to virulence as it enables the pathogen to colonize and adhere to host surfaces (O'Toole et al., 1996; Yang & Defoirdt, 2014; Finlay & Falkow, 1997). Both norepinephrine and dopamine significantly stimulated swimming motility and the expression of genes involved in flagellum synthesis (structural genes and regulators including the flagellar master regulator) in *Vibrio campbellii* (Yang et al., 2014)

Furthermore, we found that the dopamine receptor antagonist chlorpromazine can neutralize the motility-inducing effect of dopamine, and the α-adrenergic receptor antagonists phentolamine and phenoxybenzamine could neutralize the motility-inducing effect of norepinephrine, whereas the β-adrenergic receptor antagonist propranolol had no or very little effect (**Chapter 6**). Catecholamine receptor antagonists have been used extensively to identify and characterize catecholamine receptors in mammals. These results suggest that bacterial response systems for catecholamines possess a degree of specificity similar to mammalian catecholamine receptors.

Finally, based on the interesting results from the *in vitro* study, we went further to investigate the impact of both norepinephrine and dopamine and their respective receptor antagonists on the virulence of *Vibrio campbellii* towards giant freshwater prawn larvae *in vivo*. In this *in vivo* test, we tested the effect of catecholamines on

prawn larvae either if directly applied to the rearing water or if the pathogen was pretreated with catecholamines prior to inoculation into the rearing water. The pretreatment simulated transmission from a host site where catecholamines are present at high levels (e.g. due to stress and/or cell and tissue damage) to another host. It also avoided any direct effect of the compounds on the host (e.g. decreased activity of the defense system) ensuring that any impact on survival of challenged prawn larvae was due to increased virulence of the pathogen. Our *in vivo* challenge tests revealed that pretreatment of *Vibrio campbellii* with catecholamines increased the virulence of the bacterium towards giant freshwater prawn larvae without influencing the growth of the surviving larvae. However, the effect of the antagonists on the virulence of *Vibrio campbellii* was not consistent with the *in vitro* tests, in that the α-adrenergic receptor antagonists phentolamine showed an effect in two of three independent *in vivo* experiments, while the dopamine receptor antagonist chlorpromazine showed an effect in only one of three independent *in vivo* experiments. The activities of larvae-associated bacteria and different response of the larvae to the bacteria might influence the reproducibility of the experiments.

CONCLUSION

As an alternative to antibiotics, interference with pathogen-pathogen and host-pathogen signaling indeed is a promising approach to protect aquaculture animals from bacterial diseases (**Figure 26**). Focusing on the disruption of quorum sensing, bacterial cell-to-cell communication, we concluded that quorum sensing controls the virulence of *V. campbellii* towards giant freshwater prawn larvae, and that the three quorum sensing signals have a different impact on virulence towards different hosts (with HAI-1 and AI-2 being required for full virulence towards giant freshwater prawn larvae). Moreover, the use of quorum sensing inhibitors and quorum sensing signal molecule-degrading bacteria are an effective way to control disease. In addition to quorum sensing inhibition, interfering with host-pathogen signaling might also be an effective biocontrol strategy for aquaculture.

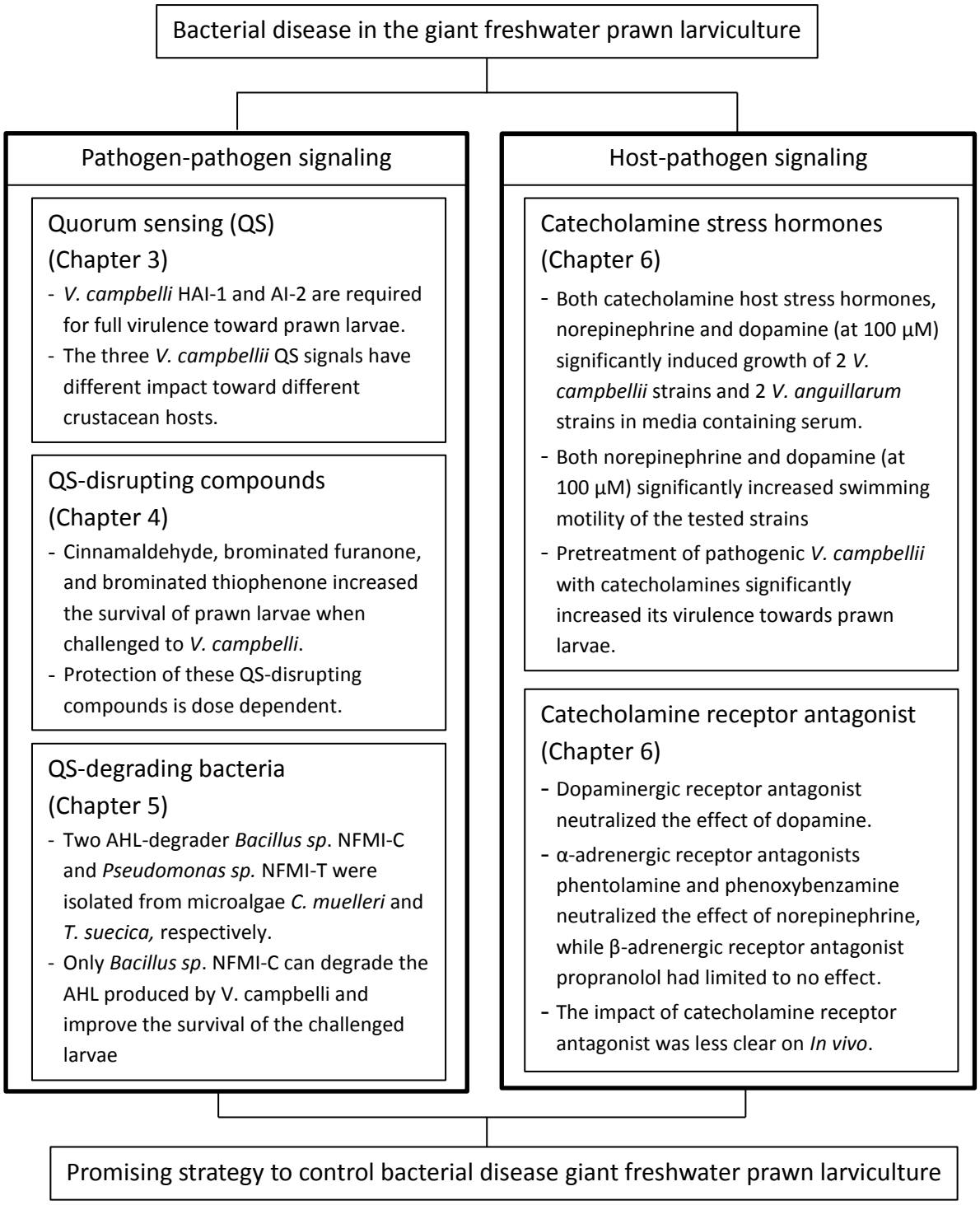


Figure 26. Schematic overview of the results from each chapter

FUTURE PERSPECTIVES

Quorum sensing has been shown to play an important role in regulating the virulence of several bacterial aquaculture pathogens, and quorum sensing inhibition has been shown to be a promising strategy to control bacterial disease in aquaculture. As an alternative to antibiotics, this strategy is considered to be non-bactericidal approach in nature (Rasko & Sperandio, 2010). Indeed, bacterial viability is usually not affected when quorum sensing is blocked (with the exception of the host-associated life phase) (Tay & Yew, 2013). Conventional antibiotics, in contrast, pose strong pressure on viability in any case where they come into contact with bacteria. Hence, when compared with antibiotics, a major advantage of quorum sensing inhibition might be a lower risk of resistance development (Defoirdt et al., 2010a; Defoirdt, 2013a). However, this will need to be confirmed in appropriate experimental conditions (*in vivo*).

This study emphasized the importance of *in vivo* experiments to prove the efficacy of quorum sensing inhibition. Although the three quorum sensing signal molecules produced by *V. campbellii* use a shared signal transduction cascade, they have a different impact on the virulence of the pathogen towards different hosts (as shown here in aquaculture pathogenic *V. campbellii* towards brine shrimp and prawn larvae). Based on this study, it seems to be necessary to verify the impact of quorum sensing-inhibiting techniques in different host-pathogen systems. Once the impact of specific quorum sensing-inhibiting techniques is known in different host-pathogens systems, they may become useful for an effective treatment of bacterial diseases in aquaculture.

Quorum sensing disruption could also be accomplished by using different kinds of signal molecule degraders recruited from natural resources (algae/bacteria/secondary metabolites/enzymes). Microalgae are able to interfere with bacterial quorum sensing by producing compounds that are able to interfere with the bacterial signal receptors and/or response regulators (Natrah et al., 2011a). For example, *Chlamydomonas reinhardtii* can produce lumichrome, a derivative of vitamin B2 (riboflavin) that has quorum sensing stimulatory activity (Rajamani et al., 2008). Another class of quorum sensing interfering compounds is the haloperoxidase enzymes which are found in

diatoms (Amin et al., 2012, Syrpas et al., 2014). It's suggested that various quorum sensing inhibitors exist in nature and still need to be explored.

In addition to pathogen-pathogen signaling, interference with host-pathogen signaling has also been proposed as a strategy to control bacterial disease. Indeed, bacteria can detect catecholamine stress hormones by the QseC receptor. Recently, a small molecule, N-phenyl-4-{[(phenylamino) thioxomethyl] amino}-benzenesulfonamide (LED209), was discovered using a high-throughput screening which inhibits the QseC mediated activation of virulence gene expression and subsequent *in vivo* virulence of various bacterial pathogens, such as enterohaemorrhagic *Escherichia coli*, *Salmonella typhimurium* and *Francisella tularensis* (Rasko et al., 2008). This compound might be useful to control bacterial disease in aquaculture because aquaculture pathogens such as *Aeromonas*, *Edwardsiella* and *Vibrio* species have been reported to respond to catecholamine stress hormones and/or to contain a QseC homologue.

Finally, many research efforts in the past decades made a significant contribution to the understanding of the mechanisms of pathogen-pathogen signaling and host-pathogen signaling. Due to rapid occurrence of antibiotic resistance, some recent works focused on inhibitory studies to address therapeutic potential for a specific sector (e.g. aquaculture). All these novel therapeutic agents need adequate trials to prove their efficacy and to exclude possible negative side effects in a real aquaculture environment ahead of their application in full scale capacity.

REFERENCES

A

Adams, T.B., Cohen, S.M., Doull, J., Feron, V.J., Goodman, J.I., Marnett, L.J., Munro, I.C., Portoghese, P.S., Smith R.L., Waddell, W.J., Wagner, B.M. 2004. The FEMA GRAS assessment of cinnamyl derivatives used as flavor ingredients. Food Chem. Toxicol. 42(2):157-185.

Alderman, D.J., Hastings, T.S. 1998. Antibiotic use in aquaculture: development of antibiotic resistance-potential for consumer health risks. Int. J. Food. Sci. Technol. 33:139-155.

Amin, S.A., Parker, M.S., Armbrust, E.V. 2012. Interactions between diatoms and bacteria. Microbiol. Mol. Biol. Rev. 76:667–680.

Austin, B., Zhang, X-H. 2006. *Vibrio harveyi*: a significant pathogen of marine vertebrates and invertebrates. Lett. Appl. Microbiol. 43:119-124.

B

Baruah, K., Cam, D.T.V., Dierckens, K., Wille, M., Defoirdt, T., Sorgeloos, P., Bossier, P. 2009. *In vivo* effects of single or combined N-acyl homoserine lactone quorum sensing signals on the performance of *Macrobrachium rosenbergii* larvae. Aquaculture 288:233-238.

Bassler, B.L. 1999. How bacteria talk to each other: regulation of gene expression by quorum sensing. Curr. Opin. Microbiol. 2:582–587.

Bassler, B.L., Greenberg, E.P., Stevens, A.M. 1997. Cross-species induction of luminescence in the quorum sensing bacterium *Vibrio harveyi*. J. Bacteriol. 179:4043-4045.

Bassler, B.L., Wright, M., Silverman, M.R. 1994. Multiple signalling systems controlling expression of luminescence in *Vibrio harveyi*: sequence and function of genes encoding a second sensory pathway. Mol. Microbiol.13:273-286.

Bearson, B.L., Bearson, S.M. 2008. The role of the QseC quorum-sensing sensor kinase in colonization and norepinephrine-enhanced motility of *Salmonella enteric* serovar Typhimurium. Microb. Pathog. 44: 271-278.

Benneche, T., Herstad, G., Rosenberg, M.L., Assev, S., Scheie, A.A. 2011. Facile synthesis of 5-(alkylidene)thiophen-2(5H)-ones: A new class of antimicrobial agents. RSC Advances 1:323–332.

Bjelland, A.M., Sørum, H., Tegegne, D.A., Winter-Larsen, H.C., Willassen, N.P., Hansen, H. 2012. LitR of *Vibrio salmonicida* is a salinity-sensitive quorum-sensing regulator of phenotypes involved in host interaction and virulence. Infect. Immun. 80:1681–1689.

Bhat, S.G., Singh, I.S.B. 1999. Vibrionaceae associated with larvae and larval rearing systems of *Macrobrachium rosenbergii*: systematics and pathogenicity. Advances in shrimp biotechnology. National Centre for Genetic Engineering and Biotechnology, Bangkok, p279.

Bi, Z.X., Liu, Y.J., Lu, C.P. 2007. Contribution of AhyR to virulence of *Aeromonas hydrophila* J-1. Res. Vet. Sci. 83:150-156.

Boon, N., De Windt, W., Verstraete, W., Top, E.M. 2002. Evaluation of nested PCR-DGGE (denaturing gradient gel electrophoresis) with group-specific 16 S rRNA primers for the analysis of bacterial communities from different wastewater treatment plants. FEMS Microbiol. Ecol. 39:101–112.

Bostock, J., McAndrew, B., Richards, R., Jauncey, K., Telfer, T., Lorenzen, K., Little, D., Ross, L., Handisyde, N., Gatward, I., Corner, R. 2010. Aquaculture: global status and trends. Phil. Trans. R. Soc. B. 365:2897-2912.

Brackman, G., Defoirdt, T., Miyamoto, C., Bossier, P., Calenbergh, S.V., Nelis, H., Coenye, T. 2008. Cinnamaldehyde and cinnamaldehyde derivatives reduce virulence in *Vibrio spp*. by decreasing the DNA-binding activity of the quorum sensing response regulator luxR. BMC Microbiol. 8(149):1-14.

Bruhn, J.B., Dalsgaard, I., Nielsen, K.F., Buccholtz, C., Larsen, J.L., Gram, L. 2005. Quorum sensing signal molecules (acylated homoserine lactones) in Gram-negative fish pathogenic bacteria. Dis. Aquat. Org. 65:43–52.

Bullen, J.J., Griffiths, E. 1999. Iron-binding proteins and host defence. In Iron and Infection. Molecular, Physiological, and Clinical Aspects. (Bullen, J.J. and Griffiths, E. eds.) John Wiley and Sons. Chichester. UK. pp 327-368.

Burridge, L., Weis, J.S., Cabello, F., Pizarro, J., Bostick, K. 2010. Chemical use in salmon aquaculture: A review of current practices and possible environmental effects. Aquaculture 306:7-23.

Byers, J.T., Lucas, C., Salmond, G.P.C., Welch, M. 2002. Nonenzymatic turnover of an *Erwinia carotovora* quorum-sensing signaling molecule. J. Bacteriol. 184:1163-1171.

C

Cabello, F.C. 2006. Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. Environ. Microbiol. 8(7):1137-1144.

Cam, D.T.V., Hao, N.V., Dierckens, K., Defoirdt, T., Boon, N., Sorgeloos, P., Bossier, P. 2009a. Novel approach of using homoserine lactone-degrading and poly-beta-hydroxybutyrate-accumulating bacteria to protect *Artemia* from the pathogenic effects of *Vibrio harveyi.* Aquaculture 291:23-30.

Cam, D.T.V., Nhan, D.T., Ceuppens, S., Hao, N.V., Dierckens, K., Wille, M., Sorgeloos, P., Bossier, P. 2009b. Effect of N-acyl homoserine lactone-degrading enrichment cultures on *Macrobrachium rosenbergii* larviculture. Aquaculture 294:5-13.

Cano-Gomez, A., Bourne, D.G., Hall, M.R., Owens, L., Hoj, L. 2009. Molecular identification, typing and tracking of *Vibrio harveyi* in aquaculture system: current methods and future prospects. Aquaculture 287:1-10.

Cao, J.G., Meighen, E.A. 1989. Purification and structural identification of an autoinducer for the luminescence system of *Vibrio harveyi*. J. Biol. Chem. 264:21670–21676.

Cavalli, R.O., Lavens, P., Sorgeloos, P. 2001. Reproductive performance of *Macrobrachium rosenbergii* female in captivity. J. World Aquaculture Soc. 32(1):60-67.

Chatterjee, A., Dutta, P.K., Chowdhury, R. 2007. Effect of fatty acids and cholesterol present in bile on expression of virulence factors and motility of *Vibrio cholerae*. Infect. Immun. 75:1946-1953.

Chen, S.C., Chen, T.H., Wang, P.C., Chen, Y.C., Huang, J.P., Lin, Y.D., Chaung, H.C., Liaw, L.L. 2003. *Metschnikowia bicuspidata* and *Enterococcus faecium* co-infection in the giant freshwater prawn *Macrobrachium rosenbergii*. Dis. Aquat. Org. 55:161-167.

Chen, X., Schauder, S., Potier, N., Van Dorsselaer, A., Pelczer, I., Bassler, B.L., Hughson, F.M. 2002. Structural identification of a bacterial quorum-sensing signal containing boron. Nature 415:545–549.

Chen, Y., Dai, J., Morris Jr, J.G., Johnson, J.A. 2010. Genetic analysis of the capsule polysaccharide (K antigen) and exopolysaccharide genes in pandemic *Vibrio parahaemolyticus* O3:K6. BMC Microbiol. 10:274.

Chen, Y.N., Fan, H.F., Hsieh, S.L., Kuo, C.M. 2003. Physiological involvement of DA in ovarian development of the freshwater giant prawn, *Macrobrachium rosenbergii*. Aquaculture 228:383-395.

Cheng, W., Chen, J.C. 2002. The virulence of Enterococcus to freshwater prawn *Macrobrachium rosenbergii* and its immune resistance under ammonia stress. Fish Shellfish Immunol. 12(2):97-109.

Chu, W., Yan J., Liu, Y., Wei, Z. 2011. Role of the quorum-sensing system in biofilm formation and virulence of *Aeromonas hydrophila*. African J. Microbiol. Res. 5:5819-5825.

Chu, W., Zhou, S., Zhu, W., Zhuang, X. 2014. Quorum quenching bacteria *Bacillus* sp. QSI-1 protect zebrafish (*Danio rerio*) from *Aeromonas hydrophila* infection. Sci. Rep. 4:5446.

Clatworthy, A.E., Pierson, E., Hung, D.T. 2007. Targeting virulence: a new paradigm for antimicrobial therapy. Nature Chem. Biol. 3:541-548.

Cogan, T.A., Thomas, A.O., Rees, L.E., Taylor, A.H., Jepson, M.A., Williams, P.H., Ketley, J., Humphrey, T.J. 2007. Norepinephrine increases the pathogenic potential of *Campylobacter jejuni*. Gut 56:1060-1065.

Croxatto, A., Chalker, V.J., Lauritz, J., Jass, J., Hardman, A.M., Williams, P., Camara, M., Milton, D.L. 2002. VanT, a homologue of *Vibrio harveyi* LuxR, regulates serine, metalloprotease, pigment, and biofilm production in *Vibrio anguillarum*. J. Bacteriol. 184:1617-1629.

D

Da silva, A. N., Canuto e Silva, J.N., Cohen, D., Issar, G. 1989. A new rickettsial diseases in *Macrobrachium rosenbergii* larvae: gross signs, cause, diagnosis and treatment. Aquaculture 2:317-337.

De Buck, E., Anne J., Lammertyn, E. 2007. The role of protein secretion systems in the virulence of the intracellular pathogen *Legionella pneumophila*. Microbiol. 153:3948–3953.

Defoirdt, T. 2007. Quorum sensing disruption and the use of short-chain fatty acids and polyhydroxyalkanoates to control luminescent vibriosis. PhD thesis, Ghent University, Belgium.

Defoirdt, T. 2013a. Antivirulence therapy for animal production: filling an arsenal with novel weapons for sustainable diseases control. PLOS Pathog. 9:e1003603.

Defoirdt, T. 2013b. Virulence mechanisms of bacterial aquaculture pathogens and antivirulence therapy for aquaculture. Rev. in Aquacult. 5:1-15.

Defoirdt, T., Benneche, T., Brackman, G., Coenye, T., Sorgeloos, P., Scheie, A.A. 2012. A quorum sensing-disrupting brominated thiophenone with a promising therapeutic potential to treat luminescent vibriosis. Plos ONE 7(7):e41788.

Defoirdt, T., Boon, N., Bossier, P. 2010a. Can bacteria evolve resistance to quorum sensing disruption?. PLoS Pathog. 6(7):e1000989.

Defoirdt, T., Boon, N., Bossier, P., Verstraete, W. 2004. Disruption of bacterial quorum sensing: an unexplored strategy to fight infections in aquaculture. Aquaculture 240:69-88.

Defoirdt, T., Boon, N., Sorgeloos, P., Verstraete, W., Bossier, P. 2007a. Alternatives to antibiotics to control bacterial infections-luminescent vibriosis in aquaculture as an example. Trends. Biotechnol. 25:472-479.

Defoirdt, T., Boon, N., Sorgeloos, P., Verstraete, W., Bossier, P. 2008. Quorum sensing and quorum quenching in *Vibrio harveyi*: lessons learned from *in vivo* work. ISME J. 2:19-26.

Defoirdt, T., Bossier, P., Sorgeloos, P., Verstraete, W. 2005. The impact of mutations in the quorum sensing systems of *Aeromonas hydrophila, Vibrio anguillarum* and *Vibrio harveyi* on their virulence towards gnotobiotically cultured *Artemia franciscana*. Environ. Microbiol. 7:1239-1247.

Defoirdt, T., Brackman G., Coenye, T. 2013a. Quorum sensing inhibitors: how strong is the evidence? Trends Microbiol. 21(12):619-624.

Defoirdt, T., Crab, R., Wood, T.K., Sorgeloos, P., Verstraete, W., Bossier, P. 2006a. Quorum sensing-disrupting brominated furanones protect the gnotobiotic brine shrimp *Artemia franciscana* from pathogenic *Vibrio harveyi*, *Vibrio campbellii* and *Vibrio parahaemolyticus* isolates. Appl. Environ. Microbiol. 72(9):6419-6423.

Defoirdt, T., Halet, D., Sorgeloos, P., Bossier, P., Verstraete, W. 2006b. Short-chain fatty acids protect gnotobiotic *Artemia franciscana* from pathogenic *Vibrio campbellii*. Aquaculture 261:804-808.

Defoirdt, T., Miyamoto, C.M., Wood, T.K., Meighen, E.A., Sorgeloos, P., Verstraete, W., Bossier, P. 2007b. The natural furanone (5*Z*)-4 bromo-5-(bromomethylene)-3-butyl-2(5*H*)-furanone disrupts quorum sensing-regulated gene expression in *Vibrio harveyi* by decreasing the DNA-binding activity of the transcriptional regulator protein luxR. Environ. Microbiol. 9:2486-2495.

Defoirdt, T., Pande, G.S.J., Baruah, K., Bossier, P. 2013b. The apparent quorum-sensing inhibitory activity of pyrogallol is a side effect of peroxide production. Antimicrobial agents and chemotherapy 57(6):2870-2873.

Defoirdt, T., Ruwandeepika, H.A.D., Karunasagar, I., Boon, N., Bossier, P. 2010b. Quorum sensing negatively regulates chitinase in *Vibrio harveyi*. Environ. Microbiol. Rep. 2:44-49.

Defoirdt, T., Sorgeloos, P. 2012. Monitoring of *Vibrio harveyi* quorum sensing activity in real time during infection of brine shrimp larvae. ISME J. 6:2314-2319.

Defoirdt, T., Sorgeloos, P., Bossier, P. 2011a. Alternatives to antibiotics for the control of bacterial diseases in aquaculture. Curr. Opin. Microbiol. 14:251-258.

Defoirdt, T., Thanh, L.D., Van Delsen, B., De Schryver, P., Sorgeloos, P., Boon, N., Bossier, P. 2011b. N-acylhomoserine lactone degrading *Bacillus* strains isolated from aquaculture animals. Aquaculture 311:258-260.

De Schryver, P., Defoirdt, T., Sorgeloos, P. 2014. Early mortality syndrom outbreaks: a microbial management issue in shrimp farming. PLoS Pathog. 10:e1003919.

Denkin, S.M., Nelson, D.R. 1999. Induction of protease activity in *Vibrio anguillarum* by gastrointestinal mucus. Appl. Environ. Microbiol. 65:3555-3560.

Dinakaran, G.K., Soundarapandian, P., Varadharajan, D. 2013. Tail rot disease in *Macrobrachium idella idella* (Hilgendorf, 1898). J. Drug Met. Toxicol. 4:155.

Dong, Y.H., Gusti, A.R., Zhang, Q., Xu, J.L., Zhang, L.H. 2002. Identification of quorum-quenching N-acyl homoserine lactonases from *Bacillus* species. Appl. Environ. Microbiol. 68(4):1754-1759.

Dong, Y.H., Wang, L.H., Xu, J.L., Zhang, H.B., Zhang, X.F., Zhang, L.H. 2001. Quenching quorum-sensing-dependent bacterial infection by an N-acyl homoserine lactonase. Nature 411:813-817.

Dong, Y.H., Wang, L.H., Zhang, L.H. 2007. Quorum-quenching microbial infections: mechanisms and implications. Philos. Trans. R. Soc.Lond. B. Biol. Sci. 362(1483):1201-1211.

Dong, Y.H., Xu, J.L., Li, X.Z., Zhang, L.H. 2000. AiiA, an enzyme that inactivates the acylhomoserine lactone quorum-sensing signal and attenuates the virulence of *Erwinia carotovora*. Proc. Natl. Acad. Sci. USA 97(7):3526-3531.

Dong, Y.H., Zhang, L.H. 2005. Quorum sensing and quorum-quenching enzymes. J. Microbiol. 43:101-109.

Donlan, R.M., Costerton, J.W. 2002. Biofilms: survival mechanisms of clinically relevant microorganisms. Clin. Microbiol. Rev. 15:167–193.

Dugan, C.C., Frakes, T.A. 1973. Culture of brackish-freshwater shrimp, *Macrobrachium acanthurus, M. carcinus* and *M. ohione*." Proceeding of. 3rd Annual Workshop, World Mariculture Society: 185-191.

E

El-Gamal, A.A., Alderman, D.J., Rodgers, C.J., Polglase, J.L., Macintosh, D. 1986. A scanning electron-microscope study of oxolinic acid treatment of burn spot lesions of *Macrobrachium rosenbergii*. Aquaculture 52(3):157-171.

F

FAO FishStat. http://www.fao.org/fishery/species/2608/en

FAO. 1988. Definition of aquaculture, Seventh Session of the IPFC Working Party of Expects on Aquaculture, IPFC/WPA/WPZ, p.1-3, RAPA/FAO, Bangkok.

FAO. 2012. The state of world fisheries and aquaculture. ISBN 978-92-5-107225-7.

FAO. 2013. Cultured Aquatic Species Information Programme. *Macrobrachium rosenbergii*. In: FAO Fisheries and Aquaculture Department [online]. Rome. http://www.fao.org/fishery/culturedspecies/Macrobrachium_rosenbergii/en [Cited 27 March 2013].

FAO. 2014. State of world fisheries and aquaculture 2014. FAO Fisheries Department. Food and Agriculture Organization in the United Nations, Rome, Italy.

Faruque, S.M., Albert, M.J., Mekalanos, J.J. 1998. Epidemiology, genetics, and ecology of toxigenic *Vibrio cholerae*. Microbiol. Mol. Biol. Rev. 62:1301-1314.

Fast, W., Tipton, P.A. 2012. The enzymes of bacterial census and censorship. Trends. Biochem. Sci. 37:7-14.

Finlay, B.B., Falkow, S. 1997. Common themes in microbial pathogenicity revisited. Microbiol. Mol. Biol. Rev. 61:136–169.

Freeman, J.A., Bassler, B.L. 1999. Sequence and function of LuxU: a two-component phosphorelay protein that regulates quorum sensing in *Vibrio harveyi*. J. Bacteriol 181:899-906.

Freestone, P. 2013. Communication between bacteria and their hosts. Scientifica 2013:1-15.

Freestone, P.P.E., Haigh, R.D., Lyte, M. 2007. Blockade of catecholamine-induced growth by adrenergic and dopaminergic receptor antagonists in *Escherichia coli* O157:H7, *Salmonella enterica* and *Yersinia enterocolitica*. BMC Microbiol. 7:8.

Freestone, P.P.E., Haigh, R.D., Williams, P.H., Lyte, M. 1999. Stimulation of bacterial growth by heat-stable, norepinephrine-induced autoinducers. FEMS Microbiol. Lett. 172:53-60.

Freestone, P.P.E, Haigh, R.D., Williams, P.H., Lyte, M. 2003. Involvement of enterobactin in norepinephrine-mediated iron supply from transferrin to enterohaemorrhagic *Escherichia coli*. FEMS Microbiol. Lett. 222:39-43.

Freestone, P.P.E., Lyte, M. 2008. Chapter 2 microbial endocrinology: experimental design issues in the study interkingdom signaling in infectious disease. Adv. Appl. Microbiol. 64:75-105.

Freestone, P.P.E., Sandrini, S.M., Haigh, R.D., Lyte, M. 2008. Microbial endrocrinology: how stress influences susceptibility to infection. Trends. Microbiol. 16(2):55-64.

Fuqua, C., Parsek, M.R., Greenberg, E.P. 2001. Regulation of gene expression by cell-to-cell communication: acyl-homoserine lactone quorum sensing. Annu. Rev. Gen. 35:439–468.

G

Galán-Vásquez, E., Luna B., Martínez-Antonio, A. 2011. The regulatory network of *Pseudomonas aeruginosa*. Microbial Informatics and Experimentation 1:3.

Galloway, W.R.J.D., Hodgkinson, J.T., Bowden, S., Welch, M., Spring, D.R. 2012. Applications of small molecule activators and inhibitors of quorum sensing in Gram-negative bacteria. Trends. Microbiol. 20:449-458.

Garcia, T., Otto, K., Kjelleberg, S., Nelson, D.R. 1997. Growth of *Vibrio anguillarum* in salmon intestinal mucus. App. Environ. Microbiol. 63:1034-1039.

Gatesoupe, F.J. 1999. The use of probiotics in aquaculture. Aquaculture 180:147-165.

Gerlach, R.G., Hensel, M. 2007. Protein secretion systems and adhesins: the molecular armory of Gram-negative pathogens. Int. J. Med. Microbiol. 297:401–415.

Gotoh, K., Kodama, T., Hiyoshi, H., Izutsu, K., Park, K.-S., Dryselius, R., Akeda, Y., Honda, T., Iida, T. 2010. Bile acid-induced virulence gene expression of *Vibrio parahaemolyticus* reveals a novel therapeutic potential for bile acid sequestrants. PLoS ONE 5:e13365.

Greenberg, E.P., Chun, C.K., Ozer, E.A., Welsh, M.J., Zabner, J. 2004. Enzymatic inactivation of a *Pseudomonas aeruginosa* quorum-sensing signal by human airway epithelia. Cell-cell Communication in bacteria (2nd), American Society for Microbiology conferences S5:1.

H

Hammer, B.K., Bassler, B.L. 2007. Regulatory small RNAs circumvent the conventional quorum sensing pathway in pandemic *Vibrio cholerae*. Proc. Natl. Acad. Sci. USA 104:11145–11149.

Han, Y. 2011. Involvement of quorum sensing in the virulence of *Edwardsiella tarda* towards culture marine fish. PhD thesis. Ghent University, Belgium.

Han, Y., Li, X., Qi, Z., Zhang, X-H., Bossier, P. 2009. Detection of different quorum-sensing signal molecules in a virulent *Edwardsiella tarda* strain LTB-4. J. Appl. Microbiol. 108:139–147.

Hargreaves, J.A. 2006. Photosynthetic suspended-growth systems in aquaculture. Aquacult. Eng. 34:344-363.

He, J., Baldini, R.L., Déziel, E., Saucier, M., Zhang, Q., Liberati, N., Lee, D., Urbach, J., Goodman, H.M., Rahme, L.G. 2004. The broad host range pathogen *Pseudomonas aeruginosa* strain PA14 carries two pathogenicity islands harboring plant and animal virulence genes. Proc. Natl. Acad. Sci. USA 101:2530-2535.

Henke, J.M., Bassler, B.L. 2004a. Quorum sensing regulates type III secretion in *Vibrio harveyi* and *Vibrio parahaemolyticus*. J. Bacteriol. 186:3794-3805.

Henke, J.M., Bassler, B.L. 2004b. Three parallel quorum sensing systems regulate gene expression in *Vibrio harveyi*. J. Bacteriol. 186:6902-6914.

Hense, B.A., Kuttler, C., Müller, J., Rothballer, M., Hartmann, A., Kreft, J.U. 2007. Does efficiency sensing unify diffusion and quorum sensing? Nat. Rev. Microbiol. 5:230-239.

Hentzer, M., Givskov, M. 2003. Pharmacological inhibition of quorum sensing for treatment of chronic bacterial infections. J. Clin. Invest. 112:1300-1307.

Heuer, O.E., Kruse, H., Grave, K., Collignon, P., Karunasagar, I., Angulo, F.J. 2009. Human health consequences of use of antimicrobial agents in aquaculture. Food Saf. 49:1248-1253.

Holmström, K., Gräslund, S., Wahlström, A., Poungshompoo, S., Bengtsson, B.E., Kautsky, N. 2003. Antibiotic use in shrimp farming and implications for environmental impacts and human health. Int. J. Food Sci. Technol. 38:255-266.

Hong, H.A., Duc, L.H., Cutting, S.M. 2005. The use of bacterial spore formers as probiotics. FEMS Microbiol. Rev. 29:813-835.

Hong, K.W., Koh, C.L., Sam, C.K., Yin, W.F., Chan, K.G. 2012. Quorum quenching revisited signal decays to signaling confusion. Sensor 12(4):4661-4696.

Hsieh, S.L., Chen, S.M., Yang, Y.H., Kuo, C.M. 2006. Involvement of norepinephrine in the hyperglycemic response of the freshwater giant prawn, *Macrobrachium rosenbergii*, under cold shock. Comparat. Biochem. Physiol. Part A 143:254-263.

Hsieh, Y.C., Liang, S.M., Tsai, W.L., Chen, Y.H., Liu, T.Y., Liang, C.M. 2003. Study of capsular polysaccharide from *Vibrio parahaemolyticus*. Infect. Immun. 71:3329-3336.

Hughes, D.T., Sperandio, V. 2008. Inter-kingdom signalling: communication between bacteria and their hosts. Nat. Rev. Microbiol. 6(2): 111–120.

I

Irianto, A., Austin, B. 2002. Probiotics in aquaculture. J. Fish Dis. 25:633-642.

Ishida, Y., Ahmed, A.M., Mahfouz, N.B., Kimura, T., El-Khodery, S.A., Moawad, A.A., Shimamoto, T. 2010. Molecular analysis of antimicrobial resistance in Gram-negative bacteria isolated from fish farms in Egypt. J. Vet. Med. Sci. 72:727-734.

Israngkura, A., Sae-Hae, S. 2002. A review of the economic impacts of aquatic animal disease. In: J.R. Arthur, M.J. Phillips, R.P. Subasinghe, M.B. Reantaso and I.H. MacRae. (eds.) Primary Aquatic Animal Health Care in Rural, Small-scale, Aquaculture Development. FAO Fish. Tech. Pap. No. 406. p. 253-286.

Iyer, L.M., Aravind, L., Coon, S.L., Klein, D.C., Koonin, E.V. 2004. Evolution of cell-cell signaling in animals: did late horizontal gene transfer from bacteria have a role? Trends Genet. 20:292–299.

J

Janssens, J.C.A., De Keersmaecker, S.C.J., De Vos, D.E., Vanderleyden, J. 2008a. Small molecules for interference with cell-cell-communication systems in Gram-negative bacteria. Curr. Med. Chem. 15:2144-2156.

Janssens, J.C.A., Steenackers, H., Robijns, S., Gellens, E., Levin, J., Zhao, H., Hermans, K., De Coster, D., Verhoeven, T.L., Marchal, K., Vanderleyden, J., De Vos, D., De Keersmaecker, S.C.J. 2008b. Brominated furanones inhibit biofilm formation by *Salmonella enterica* Serovar Typhimurium. App. Environ. Microbiol. 74(21):6639-6648

Jayaraman, A., Wood, T.K. 2008. Bacterial quorum sensing: signals, circuits, and implications for biofilms and disease. Annu. Rev. Biomed. Eng.10:145-167.

K

Kalia, V.C. 2013. Quorum sensing inhibitors: an overview. Biotechnol. Adv. 31:224-245.

Kalia, V.C., Purohit, H.J. 2011. Quenching of the quorum sensing system: potential antibacterial drug targets. Crit. Rev. Microbiol. 37:121-40.

Karunasagar, I., Pai, R., Malahti, G.R., Karunasagar, I. 1994. Mass mortality of *Penaeus monodon* larvae due to antibiotic-resistant *Vibrio harveyi* infection. Aquaculture 128: 203–209.

Kendall, M.M., Rasko, D.A., Sperandio. V. 2007. Global effects of the cell-to-cell signaling molecules autoindicer-2, autoinducer-3, and epinephrine in luxS mutant of enterohemorragic *Escherichia coli*. Infect. Immun. 75:4875-4884.

Kennedy, B., Venugopal, M.N., Karunasagar, I., Karunasagar, I. 2006. Bacterial flora associated with the giant freshwater prawn *Macrobrachium rosenbergii*, in the hatchery system. Aquaculture 261:1156-1167.

Kim, B.S., Kim, J.S. 2002. Cholesterol induce oligomerization of *Vibrio vulnificus* cytolysin specifically. Exp. Mol. Med. 34:239-242.

Kouzuma, A., Watanabe, K. 2015. Exploring the potential of algae/bacteria interactions. Curr. Opin. Biotechnol. 33:125–129.

L

Lacroix, D., Glude, J., Thomas, J.E., Le-Menn, H. 1994. Lessons from four different strategies in the development of freshwater prawn culture (*Macrobrachium rosenbergii*) in America since 1977. World Aquacult. 25(1):5-17.

Lalloo, R., Maharajh, D., Görgens, J., Gardiner, N., Görgens, J.F. 2009. High-density spore production of *B. cereus* aquaculture biological agent by nutrient supplementation. Appl. Microbiol. Biotechnol. 83:59-66.

Lalloo, R., Ramchuran, S., Ramduth, D., Görgens, J., Gardiner, N. 2007. Isolation and selection of *Bacillus* spp. as potential biological agents for enhancement of water quality in culture of ornamental fish. J. Appl. Microbiol. 103:1471–1479.

Larsen, M.H., Larsen, J.L., Olsen, J.E. 2001. Chemotaxis of *Vibrio anguillarum* to fish mucus: role of the origin of the fish mucus, the fish species and the serogroup of the pathogen. FEMS Microbiol. Ecol. 38:77-80.

LaSarre, B., Federle, M.J. 2013. Exploiting quorum sensing to confuse bacterial pathogens. Microbiol. Mol. Biol. Rev. 77(1):73-111.

Lavens, P., Sorgeloos, P. 1996. Manual on the production and use of live food for aquaculture. FAO Fisheries Technical Paper No. 361. FAO. Rome, Italy.

Leung, K.Y., Siame, B.A., Tenkink, B.J., Noort, R.J., Mok, Y.K. 2012. *Edwardsiella tarda* – virulence mechanisms of an emerging gastroenteritis pathogen. Microbes and Infection 14:26–34.

Li, J.T., Lee, P.P., Chen, O.C., Cheng, W., Kuo, C.M. 2005. Dopamine depresses the immune ability and increases susceptibility to *Lactococcus garvieae* in the freshwater giant prawn, *Macrobrachium rosenbergii*. Fish Shellfish Immunol. 19:269-280.

Li, X. 2014. The impact of cell-to-cell signalling and host cues on the virulence of *Vibrio anguillarum* towards gnotobiotic sea bass (*Dicentrarchus labrax*) larvae. PhD thesis, Ghent University, Belgium.

Li, X., Bossier, P., Dierckens, K., Laureau, S., Defoirdt, T. 2015. The impact of mucin, bile salts and cholesterol on the virulence of *Vibrio anguillarum* towards gnotobiotic sea bass (*Dicentrarchus labrax*) larvae. Vet. Microbiol. 175:44-49.

Li, X., Han, Y., Yang, Q., Zhang, X.H. 2010. Detection of quorum sensing signal molecules and mutation of luxS gene in *Vibrio ichtyoenteri*. Res. Microbiol. 161:51-57.

Li, X., Yang, Q., Dierckens, K., Milton, D.L., Defoirdt, T. 2014. RpoS and indole signaling control the virulence of Vibrio anguillarum towards gnotobiotic sea bass (*Dicentrarchus labrax*) larvae. PLoS ONE 9(10):e111801.

Lilley, B.N., Bassler, B.L. 2000. Regulation of quorum sensing in *Vibrio harveyi* by LuxO and sigma-54. Mol. Microbiol. 36:940-954.

Lin, B., Wang, Z., Malanoski, A.P., O'Grady, E.A., Wimpee, C.F., Vuddhakul, V., Alves Jr, N., Thompson, F.L., Gomez-Gil, B., Vora, G.J. 2010. Comparative genomic analyses identify the *Vibrio harveyi* genome sequenced strains BAA-1116 and HY01 as *Vibrio campbellii.* Environ. Microbiol. Rep. 2:81-89.

Lin, Y.H., Xu, J.L., Hu, J.Y., Wang, L.H., Ong, S.L., Leadbetter, J.R., Zhang, L.H. 2003. Acyl-homoserine lactone acylase from *Ralstonia* strain XJ12B represents a novel and potent class of quorum-quenching enzymes. Mol. Microbiol. 47(3):849-860.

Liu, H., Gu, D., Cao, X., Liu, Q., Wang, Q., Zhang, Y. 2012. Characterization of a new quorum sensing regulator luxT and its role in the extracellular protease production, motility, and virulence in fish pathogen *Vibrio alginolyticus*. Archives Microbiol. 194:439-452.

Lönn-Stensrud, J., Naemi, A., Benneche, T., Petersen, F.C., Scheie, A.A. 2012. Thiophenones inhibit *Staphylococcus epidermidis* biofilm formation at nontoxic concentrations. FEMS Immunol. Med. Microbiol. 65:326–334.

Lyte, M. 2004. Microbial endocrinology and infectious disease in the 21st century. Trends Microbiol. 12:14-20.

Lyte, M., Frank, C.D., Green, B.T. 1996. Production of an autoinducer of growth by norepinephrine cultured *Escherichia coli* O157:H7. FEMS Microbiol. Lett. 139:155-159.

M

Maddox, M.B., Manzi J.J. 1976. The effects of algal supplements on static system culture on *Macrobrachium rosenbergii* (de Man) larvae. Proc. World Mariculture Soc. 7:677-698.

Mah, T.F.C., O'Toole, G.A. 2001. Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiol. 9:34–39.

Manefield, M., Rasmussen, T.B., Hentzer, M., Andersen, J.B., Steinberg, P., Kjelleberg S., Givskov, M. 2002. Halogenated furanones inhibit quorum sensing through accelerated LuxR turnover. Microbiol. 148:1119–1127.

Marques, A., Dhont, J., Sorgeloos, P., Bossier, P. 2004. Evaluation of different yeast cell wall mutants and microalgae strains as feed for gnotobiotically-grown brine shrimp *Artemia franciscana*. J. Exp. Mar. Biol. Ecol. 312:115-136.

Mayali, X., Azam, F. 2004. Algicidal bacteria in the sea and their impact on algal blooms. J. Eukary. Microbiol. 51:139–144.

McCarter, L.L. 2001. Polar flagellar motility of the Vibrionaceae. Microbiol. Mol. Biol. Rev. 65:445–462.

McCarter, L.L. 2004. Dual flagellar systems enable motility under different circumstances. J. Mol. Microbiol. Biotechnol. 7:18-29.

McGrath, S., Wade, D.S., Pesci, E.C. 2004. Dueling quorum sensing systems in *Pseudomonas aeruginosa* control the production of the *Pseudomonas* quinolone signal (PQS). FEMS Microbiol. Lett. 230:27–34.

Miller, M.B., Bassler, B.L. 2001. Quorum sensing in bacteria. Annu. Rev. Microbiol. 55:165–99.

Milton, D.L., 2006. Quorum sensing in vibrios: complexity for diversification. Int. J. Med. Microbiol. 296:61-71.

Milton, D.L., Hardman, A., Camara, M., Chhabra, S.R., Bycroft, B.W., Stewart, G.S., Williams, P. 1997. Quorum sensing in *Vibrio anguillarum*: characterization of the vanI/vanR locus and identification of the autoinducer N-(3-oxodecanoyl)-L-homoserine lactone. J. Bacteriol. 179:3004-3012.

Mok, K.C., Wingreen, N.S., Bassler, B.L. 2003. *Vibrio harveyi* quorum sensing: a coincidence detector for two autoinducers controls gene expression. EMBO J. 22:870-881.

Molina, L., Constantinescu, F., Michel, L., Reimmann, C., Duffy, B., Defago, G. 2003. Degradation of pathogen quorum-sensing molecules by soil bacteria: a preventive and curative biological control mechanism. FEMS Microbiol. Ecol. 45:71-81.

Moriarty, D.J.W. 1998. Disease control in shrimp aquaculture with probiotic bacteria. In Microbial Biosystems: New frontiers. Proceedings of the Eighth International Symposium on Microbial Ecology. Bell. C.R., Brylinsky, M. and Johnson-Green, P. (eds). Halifax, NS, Canada: Atlantic Canada Society for Microbial Ecology.

Morohoshi, T., Inaba, T., Kato, N., Kanai, K., Ikeda, T., 2004. Identification of quorum-sensing signal molecules and the LuxRI homologs in fish pathogen *Edwardsiella tarda*. J. Biosci. Bioeng. 98:274–281.

Muller-Feuga, A. 2000. The role of micro-algae in aquaculture: situation and trends. J. Appl. Phycol. 12:527-534.

N

Nagpure, A., Gupta, R.K. 2013. Purification and characterization of an extracellular chitinase from antagonistic *Streptomyces violaceusniger*. J. Basic Microbiol. 53:429-439.

Nakai, T., Park, S.C. 2002. Bacteriophage therapy infectious diseases in aquaculture. Res. Microbiol. 153:13-18.

Nakano, M., Takahashi, A., Sakai, Y., Kawano, M., Harada, N., Mawatari, K., Nakaya, Y. 2007a. Catecholamine-induced stimulation of growth in *Vibrio* species. Lett. Appl. Microbiol. 44:649-653.

Nakano, M., Takahashi, A., Sakai, Y., Nakaya, Y. 2007b. Modulation of pathogenicity with norepinephrine related to the type III secretion system of *Vibrio parahaemolyticus*. J. Infect. Dis. 195:1353–1360.

Natrah, F.M.I. 2011. Role of bacterial quorum sensing and micro-algae in fish and crustacean larviculture. PhD thesis. Ghent University. Belgium.

Natrah, F.M.I., Alam, M.I., Pawar, S., Harzevili, S., Nevejan, N., Boon, N., Sorgeloos, P., Bossier, P., Defoirdt, T. 2012. The impact of quorum sensing on the virulence of *Aeromonas hydrophila* and *Aeromonas salmonicida* towards burbot (*Lota lota* L.) larvae. Vet. Microbiol. 159:77-82.

Natrah, F.M.I., Bossier, P., Sorgeloos, P., Yusoff, F.M., Defoirdt, T. 2013. Significance of microalgal-bacterial interactions for aquaculture. Rev. Aquacult. 5:1-14.

Natrah, F.M.I., Defoirdt, T., Sorgeloos, P., Bossier, P. 2011a. Disruption of bacterial cell-to-cell communication by marine organisms and its relevance to aquaculture. Marine Biotechnol. 13:109–126.

Natrah, F.M.I., Ruwandeepika, H.A.D., Pawar, S.D., Karunasagar, I., Sorgeloos, P., Bossier, P., Defoirdt, T. 2011b. Regulation of virulence factors by quorum sensing in *Vibrio harveyi.* Vet. Microbiol. 154:124-129.

Naylor, R., Burke, M. 2005. Aquaculture and ocean resources: raising tigers of the sea. Ann. Rev. Environ. Res. 30: 185-218.

Nealson, K.H., Platt, T., Hastings, J.W. 1970. Cellular control of the synthesis and activity of the bacterial luminescent system. J. Bacteriol. 104:313–322.

New, M.B. 2003. Farming freshwater prawns: a manual for the culture of the giant river prawn, *Macrobrachium rosenbergii*. FAO Fisheries Technical Paper No. 428: 145-146, FAO, Rome, Italy.

New, M.B., Nair, C.M., 2012. Review article: Global scale of freshwater prawn farming. Aquacult. Res. 43:960-969.

New, M.B. Singholka, S. 1982. Freshwater Prawn Farming: A Manual for the culture of *Macrobrachium rosenbergii*. — 116 pp. FAO Fisheries Technical Paper No 225 — FIRI/T225 (En). Food and Agriculture Organization of the United Nations. Rome, Italy

New, M.B., Valenti, W.C. 2000. Freshwater prawn culture, the farming of *Macrobrachium rosenbergii*. Blackwell Science Ltd.

New, M.B., Valenti, W.C., Tidwell, J.H., D'Abramo, L.R., Kutty, M.N. 2010. Freshwater prawn: biology and farming. Blackwell Publishing Ltd.

Ng, W.L., Bassler B.L. 2009. Bacterial quorum-sensing network architectures. Annu. Rev. Genet. 43:197-222.

Ng, W.L., Perez, L.J., Wei, Y., Kraml, C., Semmelhack, M.F., Bassler, B.L. 2011. Signal production and detection specificity in *Vibrio* CqsA/CqsS quorum-sensing systems. Mol. Microbiol. 79:1407-1417.

Nhan, D.T. 2009. Optimization of hatchery protocols for *Macrobrachium rosenbergii* culture in Vietnam. PhD thesis, Ghent University, Belgium.

Nhan, D.T., Cam, D.T.V., Wille, M., Defoirdt, T., Bossier, P., Sorgeloos, P. 2010. Quorum quenching bacteria protect *Macrobrachium rosenbergii* larvae from *Vibrio harveyi* infection. J. Appl. Microbiol. 109:1007-1016.

Njoroge, J., Sperandio, V. 2009. Jamming bacterial communication: new approaches for the treatment of infectious diseases. EMBO Mol. Med. 1:201-210.

O

O'Toole, R., Milton, D.L., Wolf-Watz, H. 1996. Chemotactic motility is required for invasion of the host by the fish pathogen *Vibrio anguillarum*. Mol. Microbiol. 19:625-637.

Oanh, D.T.T., Hoa, T.T.T., Phuong, N.T. 2001. Characterization and pathogenicity of *Vibrio* bacteria isolated from freshwater prawn (*Macrobrachium rosenbergii*) hatcheries. In proceedings of the 2001 annual workshop of JIRCAS Mekong delta project, 27-29, November 2001. CLLRI-CTU-JIRCAS, Vietnam.

Osawa, R., Arakawa, E., Okitsu, T., Yamai, S., Watanabe, H. 2002. Levels of thermostable direct hemolysin produced by *Vibrio parahaemolyticus* O3:K6 and other serovars grown anaerobically with the presence of a bile acid. Curr. Microbiol. 44: 302-305.

Ottaviani, E., Franceschi, C. 1996. The neuroimmunology of stress from invertebrates to man. Prog. Neurobiol. 48:421-440.

P

Pace, J.L., Chai, T.J., Rossi, H.A., Jiang, X. 1997. Effect of bile on *Vibrio parahaemolyticus*. Appl. Environ. Microbiol. 63:2372-2377.

Palmer, P.J., Burke, M.J., Palmer, C.J., Burke, J.B. 2007. Developments in controlled green-water larval culture technologies for estuarine fishes in Queensland, Australia and elsewhere. Aquaculture 272:1-21.

Pan, J., Ren, D. 2009. Quorum sensing inhibitors: a patent overview. Expert Opin. Ther. Patents 19:1581-1601.

Pan, L., Liu, H., Zhao, Q. 2014. Effect of salinity on the biosynthesis of amines in *Litopenaeus vannamei* and the expression of gill related ion transport genes. J. Ocean Univ. China 13:453-459.

Pande, G.S.J., Natrah, F.M.I., Sorgeloos, P., Bossier, P., Defoirdt, T. 2013a. The *Vibrio campbellii* quorum sensing signals have a different impact on virulence of the bacterium towards different crustacean hosts. Vet. Microbiol. 167:540-545.

Pande, G.S.J., Scheie, A.A., Benneche, T., Wille, M., Sorgeloos, P., Bossier, P., Defoirdt, T. 2013b. Quorum sensing-disrupting compounds protect larvae of the giant freshwater prawn *Macrobrachium rosenbergii* from *Vibrio harveyi* infection. Aquaculture 406-407:121-124

Pande, G.S.J., Suong, N.T., Bossier, P., Defoirdt, T. 2014. The catecholamine stress hormones norepinephrine and dopamine increase the virulence of pathogenic *Vibrio anguillarum* and *Vibrio campbellii*. FEMS Microbiol. Ecol. 90:761-769.

Park, S.Y., Lee, S.J., Oh, T.K., Oh, J.W., Koo, B.T., Yum, D.Y., Lee, J.K. 2003. AhlD, an N-acylhomoserine lactonase in *Arthrobacter* sp., and predicted homologues in other bacteria. Microbiol. 149:1541-1550.

Parsek, M.R., Val, D.L., Hanzelka, B.L., Cronan Jr., J.R., Greenberg, E.P. 1999. Acyl homoserine-lactone quorum-sensing signal generation. Proc. Natl. Acad. Sci. USA. 96:4360-4365.

Pascual, J., Macian, M.C., Arahal, D.R., Garay, E., Pujalte, M.J. 2010. Multilocus sequence analysis of the central clade of the genus *Vibrio* by using the 16S rRNA, recA, pyrH, rpoD, gyrB, rctB and toxR genes. Int. J. Syst. Evol. Microbiol. 60:154-165.

Pillai, D., Bonami, J. R. 2012. A review on the diseases of freshwater prawns with special focus on white tail disease of *Macrobrachium rosenbergii*." Aquacult. Res. 43(7):1029-1037.

R

Rajamani, S., Bauer, W.D., Robinson, J.B., Farrow III, J.M., Pesci, E.C., Teplitski, M., Gao, M., Sayre, R.T., Phillips, D.A. 2008. The vitamin riboflavin and its derivative lumichrome activate the LasR bacterial quorum-sensing receptor. Mol. Plant-Microbe Interact. 21: 1184–1192.

Rasch, M., Buch, C., Austin, B., Slierendrecht, J., Ekmann, K.S., Larsen, J.L., Johansen, C., Riedel, K., Eberl, L., Givskov, M., Gram, L. 2004. An inhibitor of bacterial quorum sensing reduces motalities caused by vibriosis in rainbow trout (*Oncorhynchus mykiss* Walbaum). Syst. Appl. Microbiol. 27:350-359.

Rasko, D.A., Moreira, C.G., Li, de R., Reading, N.C., Ritchie, J.M., Waldor, M.K., Williams, N., Taussig, R., Wei, S., Roth, M., Hughes, D.T., Huntley, J.F., Fina, M.W., Falck, J.R., Sperandio, V. 2008. Targeting QseC signaling and virulence for antibiotic development. Science 321:1078-1080.

Rasko, D.A., Sperandio, V. 2010. Anti-virulence strategies to combat bacteria-mediated disease. Nat. Rev. Drug. Discov. 9(2):117-28.

Rasmussen, T.B., Givskov, M. 2006. Quorum-sensing inhibitors as anti-pathogenic drugs. Int. J. Med. Microbiol. 296:149-161.

Ratledge, C., Dover, L.G. 2000. Iron metabolism in pathogenic bacteria. Annu. Rev. Microbiol. 54:881–941.

Rawn, D.F.K., Krakalovich, T., Forsyth, D.S., Roscoe, V. 2009. Analysis of fin and non-fin fish products for azamethiphos and dichlorvos residues from the Canadian retail market. Int. J. Food Sci. Technol. 44:1510–1516.

Ren, D., Sims, J.J., Wood, T.K. 2001. Inhibition of biofilm formation and swarming of *Escherichia coli* by (5Z)-4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone. Environ. Microbiol. 3:731-736.

Rengpipat, S., Rukpratanporn, S., Piyatiratitivorakul, S., Menasveta, P. 2000. Immunity enhancement in black tiger shrimp (*Penaeus monodon*) by a probiont bacterium (*Bacillus* S11). Aquaculture 191:271-288.

Roque, A., Molina-Aja, A., Bolan-Mejia, C., Gomez-Gil, B. 2001. *In vitro* susceptibility to 15 antibiotics of vibrios isolated from penaeid shrimps in Northwestern Mexico. Int. J. Antimicrob. Agents 17:383-387.

Rui, H., Liu, Q., Ma, Y., Wang, Q., Zhang, Y. 2008. Roles of LuxR in regulating extracellular alkaline serine protease A, extracellular polysaccharide and mobility of *Vibrio alginolyticus*. FEMS Microbiol. Lett. 285:155–162.

Rutherford, S.T., van Kessel, J.C., Shao, Y., Bassler, B.L. 2011. AphA and LuxR/HapR reciprocally control quorum sensing in vibrios. Genes and Development 25:397–408.

Ruwandeepika, H.A.D., Bhowmick, P.P., Karunasagar, I., Bossier, P., Defoirdt, T. 2011. Quorum sensing regulation of virulence gene expression in *Vibrio harveyi in vitro* and *in vivo* during infection of brine shrimp (*Artemia franciscana*). Environ. Microbiol. Rep. 3:597-602.

Ruwandeepika, H.A.D., Jayaweera, T.S.P., Bhowmick, P.P., Karunasagar, I., Bossier, P., Defoirdt, T., 2012. Pathogenesis, virulence factors and virulence regulation of vibrios belonging to the *Harveyi* clade. Rev. Aquacult. 4:59-74.

S

Sakr, M.M., Aboshanab, K.M.A., Aboulwafa, M.M., Hassouna, N.A.H. 2013. Characterization and complete sequence of lactonase enzyme from *Bacillus weihenstephanensis* Isolate P65 with potential activity against acyl homoserine lactone

signal molecules. Hindawi Publishing Corporation BioMed Research International Volume 2013, Article ID 192589, 10 pages http://dx.doi.org/10.1155/2013/192589.

Salanoubat, M., Genin, S., Artiguenave, F., Gouzy, J., Mangenot, S., Arlat, M., Billault, A., Brottier, P., Camus, J. C., Cattolico, L., Chandler, M., Choisne, N., Claudel-RenardI, C., Cunnac, S., Demange, N., Gaspin, C., Lavie, M., Moisan, A., Robert, C., Saurin, W. Schiex, T., Siguier, P., Thebault, P., Whalen, M., Wincker, P. Levy, M., Weissenbach, J., Bouche, C.A. 2002. Genome sequence of the plant pathogen *Ralstonia solanacearum*. Nature 415:497-502.

Samuelsen, O.B., Bergh, Ø. 2004. Efficacy of orally administered florfenicol and oxolinic acid for the treatment of vibriosis in cod (*Gadus morhua*). Aquaculture 235:27-35.

Sandifer, P.A., Hopkins, J.S., Smith T.I.J. 1975. Observations on salinity tolerance and osmoregulation in laboratory-reared *Macrobrachium rosenbergii* postlarvae (Crustacea: Caridea). Aquaculture 6:103-114.

Sandrini, S.M., Shergill, R., Woodward, J., Muralikuttan, R., Haigh, R.D., Lyte, M., Freestone, P.P.E. 2010. Elucidation of the mechanism by which catecholamine stress hormones liberate iron from the innate immune defence proteins transferrin and lactoferrin. J. Bacteriol. 192:587–594.

Sawabe, T., Kita-Tsukamoto, K., Thompson, F.L. 2007. Inferring the evolutionary history of vibrios by means of multilocus sequence analysis. J. Bacteriol. 189:7932-7936.

Schwenteit, J., Gram, L., Nielsen, K.F., Fridjonsson, O.H., Bornscheuer, U.T., Givskov, M., Gudmundsdottir, B.K. 2011. Quorum sensing in *Aeromonas salmonicida* subsp. achromogenes and the effect of the autoinducer synthase AsaI on bacterial virulence. Vet. Microbiol. 147:389-397.

Seshadri, R., Joseph, S.W., Chopra, A.K., Sha, J., Shaw, J., Graf, J., Haft, D., . Wu, M., Ren, Q., Rosovitz, M.J., Madupu, R., Tallon, L., Kim, M., Jin, S., Vuong, H., Stine O.C., Ali, A., Horneman, A.J., Heidelberg, J.F. 2006. Genome sequence of *Aeromonas hydrophila* ATCC 7966^{T}: Jack of all trades. J. Bacteriol. 188:8272-8282.

Shailender, M., Krishna, P.V., Suresh, B.C., Srikanth, B. 2012. Impact of diseases on the growth and survival of giant freshwater prawn, *Macrobrachium rosenbergii* (De Man) larvae in the hatchery level. World J. Fish Mar. Sci. 4(6):620-625

Sharaff, F., Freestone, P. 2011. Microbial Endocrinology. Central Eur. J. Biol. 6:685-694.

Skaar, E.P. 2010. The battle for iron between bacterial pathogens and their vertebrate hosts. PLoS Pathog. 6(8):e1000949.

Smith P. 2008. Antimicrobial resistance in aquaculture. Rev. Sci. Tech. Off. Int. Epiz. 27:243-264

Sørum, H. 2006. Antimicrobial drug resistance in fish pathogens. In Antimicrobial drug resistance in bacteria of animal origin. Aarestrup, F.M. (ed.). Washington, DC, USA: American Society for Microbiology Press, chapter 13:213-238.

Soto-Rodriguez, S.A., Roque, A., Lizarraga-Partida, M.L., Guerra-Flores, A.L., Gomez-Gil, B. 2003. Virulence of luminous vibrios to *Artemia franciscana* nauplii. Dis. Aquat. Org. 53:231-240.

Sperandio, V., Torres, A.G., Jarvis, B., Nataro, J.P., Kaper, J.B. 2003. Bacteria-host communication: the language of hormones. Proc. Natl. Acad. Sci. USA 100:8951-8956.

Steenackers, H.P., Levin, J., Janssens, J.C., De Weerdt, A., Balzarini, J., Vanderleyden, J., De Vos, D.E., De Keersmaecker, S.C. 2010. Structure-activity relationship of brominated 3-alkyl-5-methylene-2(5*H*)-furanones and alkylmaleic anhydrides as inhibitors of *Salmonella* biofilm formation and quorum sensing regulated bioluminescence in *Vibrio harveyi*. Bioorganic & Medicinal Chemistry 18:5224-5233.

Subasinghe, R., Bondad-Reantaso, M.G., McGladdery, S.E. 2001. Aquaculture development, health and wealth. Technical proceedings of the conference on aquaculture in the third millennium, Bangkok p167-191.

Subasinghe, R., Soto, D., Jia, J. 2009. Global aquaculture and its role in sustainable development. Rev. Aquacult. 1:2-9.

Surette, M.G., Miller, M.B., Bassler, B.L. 1999. Quorum sensing in *Escherichia coli*, *Salmonella typhimurium* and *Vibrio harveyi*: a new family of genes responsible for autoinducer production. Proc. Natl. Acad. Sci. USA 96:1639-1644.

Swift, S., Karlyshev, A.V., Fish, L., Durant, E.L., Winson, M.K., Chhabra, S.R., Williams, P., Macintyre, S., Stewart, G.S.A.B. 1997. Quorum sensing in *Aeromonas hydrophila* and *Aeromonas salmonicida*: identification of the LuxRI homologs AhyRI and AsaRI and their cognate N-acylhomoserine lactone signal molecules. J. Bacteriol. 179:5271–5281.

Swift, S., Lynch, M.J., Fish, L., Kirke, D.F., Tomas, J.M., Stewart, G.S.A.B., William, P. 1999. Quorum sensing-dependent regulation and blockade of exoprotease production in *Aeromonas hydrophila*. Infect. Immun. 67:5192-5199.

Syrpas, M., Ruysbergh, E., Blommaert, L., Vanelslander, B., Sabbe, K., Vyverman, W., De Kimpe, N., Mangelinckx, S. 2014. Haloperoxidase mediated quorum quenching by *Nitzschia cf pellucida*: study of the metabolization of *N*-acyl homoserine lactones by benthic diatom. Marine Drugs 12:352-367.

T

Taga, M.E., Bassler, B.L. 2003. Chemical communication among bacteria. Proc. Natl. Acad. Sci. USA 100 Suppl. 2:14549-54.

Tang, K., Zhang, X-H. 2014. Quorum quenching agents: resources for antivirulence therapy. Mar. Drugs 12:3245-3282.

Tay, S.B., Yew, W.S. 2013. Development of quorum-based anti-virulence therapeutics targeting gram-negative bacterial pathogens. Int. J. Mol. Sci. 14:16570-16599.

Tendencia, E., de la Pena, L.D. 2001. Antibiotic resistance of bacteria from shrimp ponds. Aquaculture 195:193-204.

Teng, S.W., Schaffer, J.N., Tu, K.C., Mehta, P., Lu, W., Ong, N.P., Bassler, B.L., Wingreen, N.S. 2011. Active regulation of receptor ratios controls integration of quorum-sensing signals in *Vibrio harveyi.* Mol. Syst. Biol. 7:491.

Thompson, F.L., Iida T., Swings, J. 2004. Biodiversity of vibrios. Microbiol. Mol. Biol. Rev. 68:403-431.

Tinh, N.T.N., Yen, V.H.N., Dieckens, K., Sorgeloos, P., Bossier, P. 2008. An acyl homoserine lactone-degrading microbial community improves the survival of first-feeding turbot larvae (*Scophthalmus maximus* L.). Aquaculture 285:56-62.

Tinh, N.T.N., Gunasekara, R.A.Y.S.A., Boon, N., Dierckens, K., Sorgeloos, P., Bossier, P. 2007. N-acyl homoserine lactone-degrading microbial enrichment cultures isolated from *Penaeus vannamei* shrimp gut and their probiotic properties in *Brachionus plicatilis* cultures. FEMS Microbiol. Ecol. 62:45-53.

Tonguthai, K. 1997. Diseases of the freshwater prawn, *Macrobrachium rosenbergii*. The Aquat. Anim. Health Res. Inst. Newsletter 4(2):1–9.

Tran, L.H., Reantaso, M., Fitzsimmons, K.M., Lightner, D.V., Hoang, P.N. 2015. Antibiotic use intended to reduce losses due to the early mortality syndrome (EMS/AHPNS) in shrimp farms in Vietnam. Proced. World Aquacult. Soc. Conf., Jeju, South Korea.

V

Vasques, J.A., Gonzales, M.P., Murado, M.A. 2005. Effect of lactic acid bacteria cultures on pathogenic microbiota from fish. Aquaculture 245:149-161.

Verbrugghe, E., Boyen, F., Gaastra, W., Bekhuis, L., Leyman, B., Parys, A.V., Haesebrouck, F., Pasmans, F. 2012. The complex interplay between stress and bacterial infections in animals. Vet. Microbiol. 155:115-127.

Verschuere, L., Rombout, G., Sorgeloos P., Verstraete, W. 2000. Probiotic bacteria as biocontrol agents in aquaculture. Microbiol. Mol. Biol. Rev. 64:655-671.

W

Wang, Q., Liu, Q., Ma, Y., Rui, H., Zhang, Y. 2007. LuxO controls extracellular protease, hemolytic activities and siderophore production in fish pathogen *Vibrio alginolyticus*. J. Appl. Microbiol. 103:1525–1534.

Wang, Y.B., Li, J.R., Lin, J. 2008. Probiotics in aquaculture: challenges and outlook. Aquaculture 281:1-4.

Wang, X., Wang, Q., Yang, M., Xiao, J., Liu, Q., Wu, H., Zhang, Y. 2011. QseBC controls flagellar motility, fimbrial hemagglutination and intracellular virulence in fish pathogen *Edwardsiella tarda*. Fish Shellfish Immunol. 30:944-953.

Waters, C.M., Bassler, B.L. 2007. The *Vibrio harveyi* quorum sensing system uses shared regulatory components to discriminate between multiple autoinducers. Genes and Development 20:2754-2767.

Whitehead, N.A., Barnard, A.M.L., Slater, H., Simpson, N.J.L., Salmond, G.P.C. 2001. Quorum-sensing in Gram-negative bacteria. FEMS Microbiol. Rev. 25:365– 404.

Wolken, W.A.M., Tramper, J., van der Werf, M.J. 2003. What can spores do for us? Trends Biotechnol. 21:338-345.

World Bank. 2013. FISH TO 2030 Prospects for Fisheries and Aquaculture. WORLD BANK REPORT NUMBER 83177-GLB.

Y

Yamaichi, Y., Duigou, S., Shakhnovich, E.A., Waldor, M.K. 2009. Targeting the replication initiator of the second *Vibrio* chromosome: towards generation of Vibrionaceae-specific antimicrobial agents. PLoS Pathog. 5:e1000663

Yang, Q., Anh, N.D.Q., Bossier P., Defoirdt T. 2014. Norepinephrine and dopamine increase motility, bioflm formation and virulence of *Vibrio harveyi*. Front. Microbiol. 5 article 584:1-12.

Yang, Q., Defoirdt, T. 2015. Quorum Sensing positively regulates flagellar motility in pathogenic *Vibrio harveyi*. Environ. Microbiol. 17(4):960–968.

Yang, M., Liu, Z., Hughes, C., Stern, A.M., Wang, H., Zhong, Z., Kan, B., Fenical, W., Zhu, J. 2013. Bile salt-induced intermolecular disulfide bond formation activates *Vibrio cholerae* virulence. Proc. Natl. Acad. Sci. USA 110: 2348-2353.

Yates, E.A., Philipp, B., Buckley, C., Atkinson, S., Chhabra, S.R., Sockett, R.E., Goldner, M., Dessaux, Y., Camara, M., Smith, H., Williams, P. 2002. N-acylhomoserine lactones undergo lactonolysis in a pH-, temperature-, and acyl chain length-dependent manner during growth of *Yersinia pseudotuberculosis* and *Pseudomonas aeruginosa*. Infect. Immun. 70:5635-5646.

Ye, J., Ma, Y., Liu, Q., Zhao, D.L., Wang, Q.Y., Zha, Y.X. 2008. Regulation of *Vibrio alginolyticus* virulence by the LuxS quorum sensing system. J. Fish Dis. 31:161–169.

Z

Zhang, H.B., Wang, L.H., Zhang, L.H. 2002. Genetic control of quorum-sensing signal turnover in *Agrobacterium tumefaciens*. Proc. Natl. Acad. Sci. USA 99:4638-4643.

Zhao, J., Chen, M., Quan, C.S., Fan, S.D. 2014. Mechanisms of quorum sensing and strategies for quorum sensing disruption in aquaculture pathogens. J. Fish Dis. 2014:1-16.

SUMMARY

SUMMARY

Aquaculture is the fastest-growing food-producing sector worldwide. One of the major aquaculture species is the giant freshwater prawn, *Macrobrachium rosenbergii*. However, diseases caused by various opportunistic pathogenic bacteria such as *Vibrio* spp. constitute a significant obstacle for the further expansion of *M. rosenbergii* cultivation. The wide and frequent use of antibiotics to control infections in aquaculture has resulted in the development and spread of antibiotic resistance. As this is gradually rendering antibiotic treatments ineffective, new strategies to control bacterial infections are needed for a sustainable further development of the aquaculture industry. Some novel strategies to control diseases have been suggested, and in this thesis, we investigated the impact of host-pathogen signaling and of quorum sensing, bacterial cell-to-cell communication, on the virulence of pathogenic bacteria towards giant freshwater prawn larvae.

Chapter 2 gives an overview of the literature describing the problems related to antibiotic usage in aquaculture, followed by a discussion of the current knowledge on major bacterial pathogenicity mechanisms in aquaculture pathogens, with a focus on host-pathogen signaling and quorum sensing.

Vibrio campbellii is one of the major pathogens of aquatic organisms, and virulence gene expression of this bacterium has been documented to be regulated by a three channel quorum sensing system. In **Chapter 3**, it is shown that although they use a shared signal transduction cascade, the signal molecules have a different impact on the virulence of the bacterium towards giant freshwater prawn larvae. Furthermore, the situation was found to be different for different hosts, emphasizing that it is highly important to study the efficacy of quorum sensing inhibitors under conditions that are as close as possible to the clinical situation.

Many bacterial aquaculture pathogens regulate virulence gene expression through quorum sensing, and consequently, quorum sensing disruption has been suggested as a novel strategy to control infections caused by these bacteria. In **Chapter 4**, we

demonstrated that the quorum sensing-disrupting compounds cinnamaldehyde, brominated furanone, and brominated thiophenone increased the survival of giant freshwater prawn larvae when challenged to pathogenic *Vibrio campbellii*.

The enzymatic inactivation of quorum sensing signal molecules produced by pathogens, such as acylhomoserine lactones, has been proposed as a novel method to combat bacterial diseases in aquaculture. In **Chapter 5,** bacterial strains that are able to degrade quorum sensing signal molecules were isolated from open micro-algal cultures. These isolates are able to establish and maintain themselves in algal cultures, degrade signal molecules produced by various aquaculture pathogens and protect giant freshwater prawn larvae from disease caused by pathogenic vibrios. Because degradation of quorum sensing signal molecules has been shown to protect different hosts from bacterial infections, these isolates could be interesting novel biocontrol strains for use in aquaculture.

In **Chapter 6**, we investigated the impact of catecholamine stress hormones on growth and virulence factor production of pathogenic vibrios *in vitro* and *in vivo*. Our *in vitro* study showed that catecholamines significantly induced growth in media containing serum (simulating the host environment) and increased swimming motility. Further, antagonists of eukaryotic catecholamine receptors were able to neutralize some of the effects of the catecholamines. Meanwhile, the *in vivo* study revealed that catecholamines significantly increased the virulence of *V. campbellii* towards giant freshwater prawn larvae. However, the impact of catecholamine receptor antagonists on *in vivo* virulence was less clear-cut when compared to the *in vitro* experiments.

Finally in **Chapter 7**, the most important results obtained in this thesis are highlighted and discussed. In conclusion, the results presented in this study showed that quorum sensing and host-pathogen signaling plays an important role in the virulence of vibrios in giant freshwater prawn larvae. Quorum sensing-disrupting compounds and quorum sensing signal molecule-degrading bacteria are promising novel tools to control bacterial

disease, and the interference with catecholamine sensing in order to control disease needs further research.

SAMENVATTING

SAMENVATTING

De aquacultuur is wereldwijd de snelst groeiende voedselproducerende sector. Eén van de belangrijkste aquacultuur species is de reuzen zoetwatergarnaal *Macrobrachium rosenbergii*. Ziekten veroorzaakt door verschillende opportunistische pathogene bacteriën zoals *Vibrio* spp. vormen echter een belangrijk obstakel voor de verdere ontwikkeling van de kweek van de reuzen zoetwatergarnaal. Het grootschalige en frequente gebruik van antibiotica om ziekten te bestrijden in de aquacultuur heeft geleid tot de ontwikkeling en verspreiding van antibioticumresistentie. Omdat dit antibioticum behandeling steeds minder doeltreffend maakt, zijn nieuwe methoden om bacteriële ziekten te bestrijden nodig voor de verdere duurzame ontwikkeling van de aquacultuur. Een aantal nieuwe strategieën om ziekten te bestrijden zijn voorgesteld in de literatuur, en in deze thesis werd de impact van gastheer-pathogeen communicatie en van quorum sensing, bacteriële cel-tot-cel communicatie op de virulentie van aquacultuurpathogenen tegen reuzen zoetwatergarnaal larven onderzocht.

Hoofdstuk 2 geeft een overzicht van de literatuur betreffende de problemen die verbonden zijn aan het gebruik van antibiotica in de aquacultuur. Vervolgens wordt de huidige kennis van de belangrijkste virulentiemechanismen in aquacultuurpathogenen besproken, met nadruk op gastheer-pathogeen en pathogeen-pathogeen communicatie.

Vibrio campbellii is één van de voornaamste pathogenen van aquatische organismen, en de expressie van virulentiegenen in deze pathogeen staat onder controle van een quorum sensing systeem dat gebruik maakt van 3 verschillende signaalmoleculen. In **Hoofdstuk 3** wordt aangetoond dat, hoewel ze een gemeesnchappelijke signaaltransductie gebruiken, de drie signaalmoleculen een verschillende impact hebben op de virulentie van de pathogeen tegenover reuzen zoetwatergarnaal larven. Bovendien vonden we dat de situatie verschillend was voor verschillende gastheren, wat aangeeft dat het belangrijk is om de effectiviteit van quorum sensing inhibitoren te bestuderen onder omstandigeden die zo goed mogelijk aansluiten bij de klinsiche omstandigheden.

Bij verschillende aquacultuurpathogenen staat de expressie van virulentiegenen onder controle van quorum sensing, en bijgevolg werd het verstoren van quorum sensing voorgesteld als een nieuwe strategie om infecties, die veroorzaakt worden door deze pathogenen, te bestrijden. In **Hoofdstuk 4** werd aangetoond dat de quorum sensing inhibitoren cinnamaldehyde, gebromeerde furanonen en gebromeerde thiofenonen de overleving verhogen van reuzen zoetwatergarnalen na challenge met pathogene *V. campbellii*.

De enzymatische inactivatie van quorum sensing moleculen, zoals acylhomoserine lactonen, werd eerder voorgesteld als een nieuwe methode om bacteriële ziekten te bestrijden in de aquacultuur. In **Hoofdstuk 5** werden bacteriestammen die in staat zijn om quorum sensing moleculen af te breken, geïsoleerd uit culturen van micro-algen. Deze isolaten zijn in staat zich te vestigen en te handhaven in algenculturen, breken de signaalmoleculen die geproduceerd worden door verschillende aquacultuurpathogenen af en beschermen reuzen zoetwatergarnaal larven tegen ziekte veroorzaakt door pathogene *Vibrio'*s. Aangezien eerder aangetoond werd dat de afbraak van quorum sensing moleculen verschillende aquatische species beschermt tegen ziekte, zouden deze isolaten interessante nieuwe bestrijdingsmiddelen kunnen zijn voor gebruik in de aquacultuur.

In **Hoofdstuk 6** werd de impact van catecholamine stresshormonen op de groei en virulentiefactor productie van pathogene *Vibrio*'s onderzocht, zowel *in vitro* als *in vivo*. Het *in vitro* onderzoek toonde aan dat catecholamines de groei van deze bacteriën stimuleerde in media die serum becatten (om het gastheermilieu na te bootsen) en dat ze tevens de mobiliteit van de bacteriën stimuleerden. Bovendien werd aangetoond dat antagonisten van eukaryotische catecholamine receptoren in staat waren om een aantal van de effecten die geïnduceerd werden door catecholamines, te neutraliseren. Het *in vivo* onderzoek toonde aan dat catecholamines de virulentie van *V. campbellii* tegenover reuzen zoetwatergarnalen vergroten. De impact van de catecholamine receptor antagonisten was echter minder duidelijk dan het geval was in de *in vitro* testen.

In **Hoofdstuk 7** worden de belangrijkste resultaten uit dit werk benadrukt en besproken. Tot besluit toonden de resultaten uit dit werk aan dat quorum sensing en gastheer-pathogeen communicatie een belangrijke rol spelen in de virulentie van *Vibrio*'s tegenover reuzen zoetwatergarnalen. Quorum sensing inhibitoren en quorum sensing molecule afbrekende bacteriën zijn veelbelovende nieuwe wapens voor de ziektebestrijding, en interferentie met de detectie van catecholamines als bestrijdingsmethode dient verder onderzocht te worden.

ACKNOWLEDGEMENT

ACKNOWLEDGEMENT

I have been greatly blessed during my PhD with opportunity to be surrounded by great people. This dissertation would not have been possible without the guidance and help of several individuals that have contributed directly or indirectly to bring this dissertation to a successful end.

My sincerest appreciations to my promoter, Prof. dr. ir. Peter Bossier for his kindly support and overall guidance in the completion of my PhD. He always available during his very busy time to give me useful feedbacks, suggestions and corrections in every aspect related to my PhD work. I considered myself very lucky to have the chance to work under supervising of a kind person and well-known professor in this field.

My deepest gratitude to my co-promoter, Dr. ir. Tom Defoirdt, a great scientist and humble supervisor. I will be forever indebted to him for his invaluable advices and his constant dedication in coaching me, he really knows how to treat a beginner like me in this QS research field and making my PhD journey such an incredible experience. A lot of things I learned from him, especially his ways of doing research, simple yet critical, direct yet precise. Thank you for being such a wonderful mentor and inspired family man. May all your future endeavors will always be filled with successes, joy and full of happiness with your wife and your two cute kids, Jasper and Pauline.

I would like to thank all the members of the reading and examination committee. Prof. dr. ir. Mia Eeckhout (chairman), Prof. Dr. ir. Sven Mangelinckx (secretary), Em. Prof. dr. Patrick Sorgeloos, Prof. dr. Annemie Decostere DVM, PhD, MSc, Dipl ECLAM, Dr. Øivind Bergh and Dr. Massimo Marzorati, thanks a lot for sharing your expertise and knowledge. I am very grateful for your critical comments and suggestions towards enhancing the manuscript.

I would like to acknowledge the government of Republic of Indonesia through Directorate general of higher education (DIKTI) for providing three years PhD scholarship and Bijzonder Onderzoeksfonds (BOF)-special research fund for finalizing PhD from Ghent University to complete my PhD-research program.

My moments in ARC with my colleagues will be cherished forever. Marc Verschraeghen, thank you for helping me with the administrative things from the beginning of my PhD until the final thesis and defense preparation. Tom Baelemans, you have been a super great officemate, a high dedicated worker of ARC, thanks for always being there for me whenever I need a friend to talk to. The secretariat crew, Alex Pieters and Caroline Van Geeteruyen, thanks for all your help during my stay in ARC. The *Macrobrachium* team, Mathieu Wille and Jorg Desmyter, thanks for your kind help to guide me during experiment with our lovely *Macrobrachium* larvae. Brigitte Van Moffaert, Anita De Haese, Geert Vandewiele and Christ Mahieu, thanks for always available to help me during my labwork in ARC. For all ARC staff, Prof. Gilbert Van Stappen, Jean Dhont, Nancy Nevejan, Mieke Eggermont, Aäron Plovie, Peter De Schryver, Kristof Dierckens, Margriet Drouillon, Kenny Vandenbrouck, 'Michael' Niu Yufeng, Kartik Baruah and Parisa, thank for a good friendship, I'm going to miss all of you.

I would like to thank all my PhD colleagues, past and present, for making the journey of doing PhD an 'unlonely' one. To the people who collaborated with me, my 'scientific partner' in QS experiment, Qian Yang, thank you for always being a good friend for sharing both scientific and non-scientific things, I always waiting for your homemade dumplings. Natrah Fatin Mohd Ikhsan, thank you for sharing of all scientific things and your kind cooperation for the preparation of our published papers. Truong Quoc Thai, thanks for your help during my *in vivo* challenge test with *Macrobrachium* larvae. PhD fellows batch 2011 Nguyen Van Hung, Nguyen Thi Xuan Hong, Joseph Laranja, Gladys Ludevese, Eamy Nursaliza and Bai Nan, thank you for the nice moments in ARC. The past and present PhD and exchange students in ARC, Sofie Vanmaele, Hu Bing, Li Xuan, Nguyen Duy Hoa, Maria Lourdes Cobo, Ramin Manafar, Mohamed Magsodi, Spyros Nikolakakis, Montakan Tamtin, Nguyen Thi Hong Van, Huyn Thanh Toi, Phung Bay, Leticia Fernadez, Andrei Baikov, Jiaojiao Zhang, Guannan Ma, Linda Xing, Dipesh Debnath, Dechamma Sony Mundanda, Rajeish Moger, Sanjay Kumar Gupta, Swati Sonkawade, Duy Le, Nguyen Viet Dung, Ruy Santos, Navid Pormehr, and Bárbara Hostins, thanks for making my life more colourful, and all the best for all of you!

During this PhD study, I experienced some events, such as joining conferences and training schools in other institutions which was really helpful to open a broader networking opportunity. Therefore, I would like to address a sincere appreciation to Dr. Luis Conceicao and Dr. Sofia Engrola (University of Algarve, Portugal), Michael New (The freshwater prawn ambassador and the past president of World Aquaculture Society), Prof. Ronaldo Oliveira Cavalli (Universidade Federal Rural de Pernambuco, Recife, Brazil), Prof. Ana Otero (University of Santiago de Compostela, Spain), the participants of Promicrobe 2012 (Nancy Xu, Phuoc, Ruy, Wanna, Giannina, Gustavo, etc), and the participants of Larvita 2012 (Nguyen Phuoc Thuong, Jose Antonio Matta, Andre Santos, Carmen Navarro, Alexandra Morgado, Andrea Franke, Kruno Bonacic, Sune Riis Sørensen, Antonis Chalaris, Bridie Grant, etc).

My Indonesian fellow PhD and Master students in the ARC lab, Magdalena Lenny Situmorang, Julie Ekasari, Romi Novriadi, Linayati, Apriana Vinasyiam, Syifania Hanifah Samara, Rosmawanti Fadjrin and Zarah Fazri Khotimah, thank you very much for your support. Having lunch, cooking, and sharing thoughts and laugh with you guys have always been a good moments during my stay in Gent.

Not to forget, Dr. Ana Bossier, thanks for invite me and friends to taste a good food with a motherly touch. I really enjoyed your cooking, especially that the tasty chicken heart.

I thank Dr. Gede Suantika, for introducing me to ARC and Ghent University, and his encouragement to pursue my study here. To all teachers in my life, the lessons I learned during my academic and professional career have become an important background for this PhD work, and I thank you all for that.

The Indonesian people and students in Ghent, Wa An and Wa Francis, Ko Jul, Ci Lanny, Aswin and Hans Kusmanto, OBSG'ers Ahmad Faizal and family, Arifin and Arima, Pak Romy, Mas Nanang Suprayogi and family, Wahyu Wijaya, Mb Mirni, Dimas Rahadian, my best friends Nuri Nurlaila Setiawan, Chobi and Okky Amalia, and all of the members of Indonesian student association in Gent (IndoSAG/PPI Gent), thank you very much for your supports during my stay in Gent.

To my master students (Nguyen Thao Suong and Uday Kumar) who shared the joy and ups and downs of doing the research together with me, I am really glad to have such dedicated students. Thank you!

I am also greatly indebted to Udayana University (Unud) and Indonesian Ministry of Higher Education for giving me the opportunity to pursue my study abroad. I would like to thank the former rector of Unud, Prof. Dr. dr. I Made Bakta, Sp.PD (KHOM), the present rector Prof. Dr. dr Ketut Suastika, Sp.PD-KEMD, dean of the faculty of mathematic and life sciences, AAG. Raka Dalem, M.Sc. dean of the faculty of marine science and fisheries, Prof. Ir. Wayan Arthana, MS, PhD, head of biology department, Drs. AA Ketut Darmadi, M.Si, and head of aquatic resource management department, Ir. I Wayan Restu, M.Si, my heartfelt thanks for encouraging me to study abroad and endless supports throughout the journey.

To the family of Biology Department, FMIPA Unud (Drs. IB Suaskara, M., Si., Dwi Ariani Yulihastuti, S.Si., M.SI., Dr. Eswaryanti Kusuma Yuni., Yan Ramona, PhD., Dr. Retno Kawuri, Dr. IB Darmayasa, Dr. Ida Ayu Astarini, Dr. Made Pharmawati, Dr. FX Sudaryanto, Dr. Intan Wiratmini, Dr. Iriani Setyawati, Dr. Watiniasih, Dra. Wayan Sudatri, Pak Job Nico Subagio, Pak Ketut Winantera and others), and the family in the faculty of marine science and fisheries, Unud (Dr. Gede Karang, Dr. Gede Hendrawan, Bu Devi Ulinuha, Pak Ima Yudha Perwira, Bu Dati Pertami, Bu Endang Suryaningtyas and Pak Budi Dwi Wiyanto). Thank you for the encouragements. I am looking forward to meet all of you again. I can’t wait to work with all of you when I’m back!

I would like to thank the former Indonesian Ambassador to the Kindom of Belgium, Grand-Duchy of Luxembourg and The European Union, Mr. Arif Havas Oegroseno, and all the staff of the Information, social-cultured and public diplomacy section, and protocol and consular section as well as the PPLN Brussels 2014 team, Pak Baktiar Hasan, dr. Gunawan Setyabudi, Ibu Noerlena Pertiwi, and Mb Irene Handayani, Pak Bonanza Taihitu, Pak Arief Dermawan, and Pak Adrianus for all the support during my stay in Belgium.

I was lucky that I live in OBSG (Oetmoeting Buitenlandse Studenten Gent), a nice and friendly place for students from developing countries who study in Gent. I would like to thank Father Charles de Hemptinne, Isabelle Mrozowski, Annemie Derbaix, Marleen Van Stappen, Phillip and Claudia for all the service and accommodation during my stay in Gent.

I would like also to thank all the people who involve related to Dikti scholarship matters, Mb Citra Amitiurna, Mb Ayus Rahmadhani, Bu Pande Dewi Jayanti, Pak Putu Wira Subagia, Devi Udayani, Grup beasiswa Dikti-S2/S3 luar negeri, Pak Diasana Putra, Bu Yudantini, Bli Pande Januraga, Dr. dr Dwi Fatmawati and all Dikti-ers Unud. Thanks for all your kind help.

Although we're a very small community, I enjoyed spending time with my Balinese family in Belgium and Nederland. Bli Made Agus Wardana and family, and Pak Putu Juli Ardika and family in Brussels, Mbok Rai, Johan and Laysa in Ghent, Bli Wayan Sudiarta and family in Dendermonde, Mb Ria Cahyani and family in Lokeren, Bli Putu Taplet Astawan and family in Stekene, Wayan Werdhi and family in Rotterdam and all of the members of Balinese Hindu community Banjar Santi Dharma Belgia. Will definitely miss the times we spent together, especially for 'ngayah' at Pura Agung Santi Bhuwana, Pairi Daiza, Belgium.

Finally, I would like to dedicate this thesis to my wife Nyoman Diah Witari, and my children, Gde Diaz Divayana and Made Deniz Indrayana who have been patiently awaiting over 4 years of my PhD-research work. My wife has worked hard to take care of our children and sacrificed to stand for lonely life during the time I have not been at home. Behind the success of my PhD-research work as well as the success of my life was always her silent sacrifices. Last but not least, great thanks to my parents, my parents in law, my brother and my sister who always supported me and expected me to finish my PhD degree. I know that words are not enough to express my gratitude to them. This thesis is a present for them. Thank you from the bottom of my heart…

CURRICULUM VITAE

Curriculum Vitae

PANDE GDE SASMITA JULYANTORO
(Pande GSJ)

PERSONAL DETAILS

Current address	:	Department of Aquatic Resources Management, Faculty of Marine Science and Fisheries, Universitas Udayana Bukit Jimbaran Campus, 80361 Bali-Indonesia
Email	:	pande_sasmita@yahoo.com
Date of birth	:	July 26th, 1980

EDUCATION

2011 - 2015 : PhD – Applied Biological Science (Aquaculture)
Lab. of Aquaculture and *Artemia* Reference Centre (ARC),
Department of Animal Production,
Faculty of Bioscience Engineering, Ghent University, Belgium

Thesis	:	The impact of pathogen-pathogen and host-pathogen signaling in larviculture of the giant freshwater prawn (*Macrobrachium rosenbergii*)
Promoter I	:	Prof. dr.ir. Peter Bossier
Promoter II	:	Dr.ir. Tom Defoirdt

2004 – 2006 : Master of Science (Biology) Laboratory of Aquatic Ecology,
School of Life Science and Technology (SITH),
Institut Teknologi Bandung (ITB), Indonesia

Thesis	:	Development of zero-water discharge technology and application of nitrifying bacteria on nursery phase of giant freshwater prawn *Macrobrachium rosenbergii* (de Man)
Supervisor	:	Dr. Gede Suantika

1998 – 2003 : Bachelor of Science (Biology), Department of Biology,
Faculty of Mathematics and Natural Sciences,
Institut Teknologi Bandung (ITB), Indonesia

Thesis	:	The impact of shelter used on stocking density and survival of giant freshwater prawn *Macrobrachium rosenbergii* (de Man) at laboratory scale.
Supervisor I	:	Dr. Noorsalam R. Nganro
Supervisor II	:	Dr. Gede Suantika

PUBLICATIONS (International peer-reviewed)

Pande GSJ, FMI Natrah, AVB Flandez, U. Kumar, Y. Niu, P. Bossier, T. Defoirdt. 2015. Isolation of AHL-degrading bacteria from micro-algal cultures and their impact on algal growth and on virulence of *Vibrio campbellii* to prawn larvae. *Applied Microbiology and Biotechnology*. In press.

Pande GSJ, NT Suong, P. Bossier, T. Defoirdt. 2014. The catecholamine stress hormones norepinephrine and dopamine increase the virulence of pathogenic *Vibrio anguillarum* and *Vibrio campbellii*. *FEMS Microbiology Ecology* 90:761-769.

Pande GSJ, FMI Natrah, P. Sorgeloos, P. Bossier, T. Defoirdt. 2013. The *Vibrio campbellii* quorum sensing signals have a different impact on virulence of the bacterium toward different crustacean hosts. *Veterinary Microbiology* 167:540-545.

Pande GSJ, AA Scheie, T. Benneche, M. Wille, P. Sorgeloos, P. Bossier, T. Defoirdt. 2013. Quorum sensing-disrupting compounds protect larvae of the giant freshwater prawn *Macrobrachium rosenbergii* from *Vibrio harveyi* infection. *Aquaculture* 406-407: 121-124.

Defoirdt T., **GSJ Pande**, K. Baruah, P. Bossier. 2013. The apparent quorum sensing inhibitory activity of pyrogallol is a side effect of peroxide production. *Antimicrobial agents and chemotherapy* 57(6):2870.

PROCEEDINGS (International conference)

Pande GSJ, Natrah FMI, U Kumar, P. Bossier, T. Defoirdt. 2015. AHL-degrading bacteria isolated from microalgae protect prawn from *Vibrio campbellii* infection. I International Symposium on Quorum Sensing Inhibition. University of Santiago de Compostela, Spain. 3-5 June 2015 (oral presentation)

Pande GSJ, NT Suong, P. Bossier, T. Defoirdt. 2014. The impact of the catecholamine stress hormones norepinephrine and dopamine on the virulence of aquaculture pathogenic *Vibrio campbellii*, Belgian Society for Microbiology Meeting, Academy Palace Brussels, Belgium, 18 November 2014 (poster presentation).

Pande GSJ, FMI Natrah, P. Sorgeloos, P. Bossier and T. Defoirdt, 2014.The three quorum sensing signals of *Vibrio campbellii* have a different impact on its virulence towards different crustacean hosts. European Aquaculture 2014, San Sebastian, Spain, 15-17 October 2014 (oral presentation).

Defoirdt T, Q Yang, **GSJ Pande**, NDQ Anh, NT Suong, P. Bossier, 2014. Impact of catecholamine stress hormones on the virulence of *Vibrio campbellii*. *Vibrio* 2014. Edinburgh, United Kingdom, 1-4 April 2014 (Presented by TD)

Pande GSJ, P. Bossier, T. Defoirdt, 2013. The impact of *Vibrio harveyi* quorum sensing and quorum sensing inhibitors on larviculture of the giant freshwater prawn *Macrobrachium rosenbergii*. Asian-Pacific Aquaculture 2013, Ho Chi Minh City, Vietnam, 11-13 December 2013 (oral presentation).

Pande GSJ, P. Bossier, T. Defoirdt, 2013. The impact of quorum sensing-disrupting compounds on survival and growth of giant freshwater prawn (*Macrobrachium rosenbergii*) larvae. Larvi 2013, Ghent, Belgium, 3-5 September 2013 (poster presentation)

Pande GSJ and AAG. Raka Dalem, 2010. The distribution of marine and freshwater captured crustacea in Bali, Indonesia. International meeting of association for tropical biology and conservation (ATBC). Sanur Beach Hotel, Bali, Indonesia, 19-23 July 2010 (oral presentation).

Pande GSJ, 2010. The development and application of ultrafiltration technology on aquaculture: Harvesting and concentrating microalgae for larviculture purposes. International bioscience and biotechnology conference, Universitas Udayana, Bali, Indonesia, 23-24 September 2010 (oral presentation).

Suantika G. and **GSJ Pande**, 2009. Development of zero-water discharged technology and nitrifying bacteria application in nursery phase of the giant freshwater prawn *Macrobrachium rosenbergii* (de Man). World Aquaculture 2009. World Trade Center, Veracruz, Mexico, 25-29 September 2009. (Presented by GS)

Pande GSJ and IG. Wenten, 2005. Application of ultrafiltration membrane in shrimp aquaculture: from lab to full scale capacity. ICOM 2005. Seoul, South Korea, 21-26 August 2005. (poster presentation).

Pande GSJ and IG Wenten, 2005. Application of ultrafiltration for total virus and bacterial removal in shrimp aquaculture. Proceeding of 3th Regional Symposium of Membrane Science and Technology 2005. Aula Barat & Timur Institut Teknologi Bandung, Indonesia, 26-27 April 2005 (oral presentation).

PROCEEDINGS (national conference)

Pande GSJ dan IG. Wenten, 2005. Membran ultrafiltrasi untuk penyisihan sempurna virus dan bakteri dalam system akuakultur udang windu (*Penaeus monodon*). Prosiding Seminar Nasional Rekayasa Kimia dan Proses 2005. JurusanTeknik Kimia. Universitas Diponegoro. Semarang.

Pande GSJ, NR. Nganro dan G. Suantika, 2004. Pengaruh penggunaan shelter terhadap tingkat kesintasan dan padat tebar udang galah dalam skala laboratorium. Prosiding Seminar Nasional MIPA IV. Institut Teknologi Bandung.

Pande GSJ, IG. Wenten dan G. Suantika, 2004. Pengembangan teknologi ultrafiltrasi untuk pemekatan mikroalga. Prosiding Seminar Nasional Rekayasa Kimia dan Proses 2004. Jurusan Teknik Kimia. Universitas Diponegoro. Semarang.

AWARDS/GRANTS

Bijzonder Onderzoeksfonds (BOF)-special research fund for finalizing PhD from Ghent University for a periods of 12 months for doctoral project entitled 'The impact of bacteria-bacteria and host-bacteria signaling on larviculture of giant freshwater prawn *Macrobrachium rosenbergii*'. Code : 01DI0114.

LARVANET COST Action Grant for participation in LARVITA Training School: Critical success factors for fish larval production in European Aquaculture, a multidisciplinary network, CCMAR/University of Algarve, Faro, Portugal 5-7 December 2012. Reference code: COST-TS-ECOST-TRAINING_SCHOOL-FA0801-191112-022991.

Doctoral scholarship from Directorate General of Higher Education of Indonesia/DIKTI for 36 months (October 2011 - October 2014).

The young researcher grant from Universitas Udayana, 2011

The young researcher grant from Universitas Udayana, 2010

WORK EXPERIENCES

2012-2014
Assistant for practical course of MSc students at Lab. Aquaculture and ARC: Disease in Aquaculture and Algae culture

2014
Video conference lecturer about the disruption of bacterial quorum sensing application in aquaculture, Sekolah Tinggi Perikanan (STP) Sorong, Papua, Indonesia.

2012 – present
Lecturer at Department of Aquatic Resources Management, Faculty of Marine Science and Fisheries, Universitas Udayana, Bali, Indonesia.

2008 – 2012
Lecturer at Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Udayana, Bali, Indonesia.

2006-2008
Hatchery staff at aquaculture private company (CP Group), Situbondo, East Java, Indonesia.

2004-2006
Junior researcher at downstream processing laboratory, Research center of Biotechnology, PAU-ITB, Bandung, West Java, Indonesia.

SUPERVISION

2014

Uday Kumar (India) – The impact of pathogen-pathogen and host-pathogen signaling on larviculture of giant freshwater prawn. Master of Science in Aquaculture at the Faculty of Bioscience Engineering, Ghent University, Belgium.

2013

Nguyen Thao Suong (Vietnam) – The impact of host stress hormones in the larvae of giant river prawn (*Macrobrachium rosenbergii*). Master of Science in Aquaculture at the Faculty of Bioscience Engineering, Ghent University, Belgium.

2010-2011

Thesis supervision of some bachelor students at Department of Biology, Faculty of Mathemathics and Natural Sciences, Universitas Udayana, Bali, Indonesia.

WORKSHOP/TRAINING/COURSES

FTNLS symposium 'Microbial Evolution: theory, simulation and experiment', KU Leuven, 6-7 May 2015.

Ecological application of biomarkers in aquatic food web studies, Department of Biology, Ghent University, Belgium, 28 January 2013 – 1 February 2013.

LARVITA 2012 training course. Critical success factors for fish larval production in European Aquaculture, a multidisciplinary network (LARVANET). CCMAR, University of Algarve, Faro, Portugal 5-7 December 2012.

PROMICROBE 2012 training course. Microbial community management in aquaculture. Ghent University, Belgium 20-22 August 2012.

UGent Doctoral School courses (2012-2015):

Effective scientific communication, Conference skills, Communication skills
The Low Countries studies, project management, leadership foundation.

SOCIAL ACTIVITIES

Member of Indonesia aquaculture society/MAI 2010 - present

Committee of Indonesian general election in Belgium/PPLN 2014 for electing the representative council, president and vice president of Indonesia 2014-2019.

Chairman of Indonesian student organization in Ghent (PPI Gent) 2013-2014.

Chairman of Indonesian cultural event 'Sounds of Heritage (SOH) 2013', De Terminal building, UGent, 11 May 2013.

Member of Balinese Hindu community and Balinese traditional music group 'saling asah' in Belgium 2011-2015.